PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 11 July 2001 (11.07.01)	
International application No. PCT/US00/22583	**Applicant's or agent's file reference** GMV-003.25
International filing date (day/month/year) 17 August 2000 (17.08.00)	**Priority date** (day/month/year) 25 August 1999 (25.08.99)
Applicant NICOLAU, Yves, Claude et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

23 March 2001 (23.03.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer H. Zhou
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

REC'D 19 OCT 2001

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
GMV-003.25		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/22583	17/08/2000	25/08/1999
International Patent Classification (IPC) or national classification and IPC A61K31/575		
Applicant GMP COMPANIES, INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
23/03/2001	17.10.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Herz, C Telephone No. +49 89 2399 8275

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/22583

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-48 as originally filed

Claims, No.:

1-49 as originally filed

Drawings, sheets:

1-2 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-49
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-49
Industrial applicability (IA)	Yes:	Claims	1-49
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

1. The Applicant is informed that this Opinion is based on the documents retrieved by the search. Due to the fact that this search was not carried out completely for all claims, since the cope of the claims are not clearly formulated this Examination Report cannot be complete.

2. From the state of the art as represented by the documents cited in the International Search Report numerous compounds which are similar in structure to compounds falling under the definition of Claim 11 are known to enhance the oxygen delivery in mammals.

Taking into account these facts the man skilled in the art would have to expect oxygen delivery enhancing capability without affecting their basic capabilities when modifying the basic moiety and/or the substituents of the groups of compounds disclosed in the state of the art. Thus representing only predictable effects the compounds claimed are considered to be obvious.

Consequently, Claims 1 to 49 are lacking inventive step under Article 33 (3) PCT.

3. The terms "lipophilic water-soluble molecule" and "ligand for a mammalian cellular receptor" are totally inappropriate in defining chemical compounds. The claims lack clarity (Article 6 PCT) since an attempt is made to define the compounds by reference to a result to be achieved rather than specifying their chemical structure by indicating the identity and number of the atoms involved.

Furthermore, the claims cover all products having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products. In the present case, the claims so lack support, and the application so lacks disclosure.

With regard to the universe of different compounds falling under the scope of Claim 11 it is noted that a technical effect which justifies the selection of the claimed compounds must be one which can be assumed to be produced by substantially all the selected compounds. The numerous non-, mono- and polycyclic isocyclic and heterocyclic moieties being further substituted by numerous different groups as given in Claim 11 virtually encompass such an enormous number of compounds and that is by now way credible that all these variants represent solutions to the problem of providing improved α-ammonium ketones as photoinitiators. There is no proven common general knowledge to show that the type of substituents that may be present in the claimed compounds would be irrelevant to the presence of the alleged properties.

For these reasons, and on the basis of what evidence there is in the case (only two actual working examples), the International Preliminary Examining Authority is not satisfied that substantially all compounds being claimed are likely to exhibit the alleged superior photoinitiating activity. Only those of the claimed compounds could possibly involve an inventive step which could be accepted as solutions of the technical problem of providing further compounds which

possess this property (Article 33 (3) PCT).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
GMV-003.25		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 00/ 22583	17/08/2000	25/08/1999
Applicant		
GMP COMPANIES, INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 8 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   AGENTS FOR THE ENHANCED OXYGEN DELIVERY IN MAMMALS

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/22583

Box I Observati ns where certain claims wer found unsearchable (C ntinuati n of item 1 f first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1 to 49 relate to compounds defined (inter alia) by reference to the following parameters: "a cationic, lipophilic, water-soluble molecule" and "an anionic ligand for a cellular receptor". The use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible. Consequently, the search has been restricted to the compounds BGTC, BGSC and IHP.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/22583

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/575 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 338 916 A (INSTITUT MERIEUX) 25 October 1989 (1989-10-25) claims 1-34	1-49
Y	WO 97 42819 A (A. HACES) 20 November 1997 (1997-11-20) claims 1-19	1-49
Y	WO 98 39358 A (GENTA INC.) 11 September 1998 (1998-09-11) claims 1-38	1-49
Y	WO 98 39359 A (GENTA INC.) 11 September 1998 (1998-09-11) claims 1-41	1-49

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 January 2001	12.02.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Herz, C

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 452 055 A (HEWLETT-PACKARD CO.) 16 October 1991 (1991-10-16) claims 1-16	1-49
Y	US 5 612 207 A (Y. C. NICOLAU ET AL.) 18 March 1997 (1997-03-18) column 23, line 15 -column 24, line 25	1-49
Y	US 5 927 283 A (D. J. ABRAHAM, M. GERBER) 27 July 1999 (1999-07-27) claims 1,2; tables 1-7	1-49
Y	US 5 872 282 A (D. J. ABRAHAM ET AL.) 16 February 1999 (1999-02-16) tables 1-7	1-49
Y	US 5 110 909 A (E. DELLACHERIE ET AL.) 5 May 1992 (1992-05-05) claims 1-23	1-49
Y	US 4 921 997 A (I. LALEZARI, P. LALEZARI) 1 May 1990 (1990-05-01) table 1	1-49
Y	US 5 079 337 A (M. LEONARD ET AL.) 7 January 1992 (1992-01-07) claims 1-29	1-49
Y	PATENT ABSTRACTS OF JAPAN vol. 017, no. 119, 12 March 1993 (1993-03-12) & JP 04 300838 A (TERUMO CORP.), 23 October 1992 (1992-10-23) abstract	1-49
Y	E. ANTONINI ET AL.: "The Effect of Anions and Cations on the Oxygen Equilibrium of Human Hemoglobin" OXYGEN AFFINITY HEMOGLOBIN RED CELL ACID BASE STATUS, PROC. ALFRED BENZON SYMP., 4TH (25YHA4), 1972, pages 121-130, XP000944291 figures 1-4	1-49
Y	N. OUDHIRI ET AL.: "Guanidinium-cholesterol cationic lipids: novl reagents for gene transfection and perspectives for gene therapy" BIOGENIC AMINES, vol. 14, no. 5, 1998, pages 537-552, XP000944064 figure 1	1-49

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	W. J. FANTL ET AL.: "Specifically Carboxymethylated Hemoglobin as an Analogue of Carbamino Hemoglobin" J. BIOL. CHEM., vol. 262, no. 26, 1987, pages 12700-12713, XP000943847 page 12700 ---	1-49
Y	K. UCHIDA ET AL.: "Effect of an Allosteric Modifier of Hemoglobin, RSR-4, on Oxygen Affinity and Oxygen Saturation of Hemoglobin in Rabbits" JPN. J. PHYSIOL., vol. 48, no. 6, 1998, pages 439-444, XP000943772 table 2 ---	1-49
Y	I. PAPASSOTIRIOU ET AL.: "Synthesized allosteric effectors of the hemoglobin molecule: A possible mechanism for improved erythrocyte oxygen release capability in hemoglobinopathy H disease" EXPERIMENTAL HEMATOLOGY, vol. 26, no. 10, 1998, pages 922-926, XP000943784 figures 1-4; tables 1,2 ---	1-49
Y	I. TABUSHI ET AL.: "Artificial Allosteric Molecules - Especially Focusing upon Allosteric O2 Binding Molecules of the Hemoglobin Type" MOL. STRUCT. ENERG., vol. 10, 1988, pages 195-218, XP000943789 tables 7-6 ---	1-24
Y	C. NADOLNY ET AL.: "Specific Interactions of the Allosteric Effector 2,3-Bisphosphoglycerate with Human Hemoglobin - A Difference FTIR Study" BIOL. CHEM. HOPPE-SEYLER, vol. 374, no. 6, 1993, pages 403-407, XP000943782 * entire document * ---	1-49
Y	K. S. KILGORE ET AL.: "RSR13, a Synthetic Allosteric Modifier of Hemoglobin, Improves Myocardial Recovery Following Hypothermic Cardiopulmonary Bypass" CIRCULATION, SUPPL., vol. 100, no. 19, 1999, pages 351-356, XP000943791 figures 1-4; tables 1,2 --- -/--	1-49

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	D. L. CURRELL ET AL.: "Synthetic polyphosphonates, polyphosphates, and phosphonocarboxylates as allosteric effectors of hemoglobin" PHOSPHORUS, SULFUR SILICON RELAT. ELEM., vol. 51-52, no. 1-4, 1990, pages 35-38, XP000943807 tables I-IV	1-49
Y	A. N. CHUVILIN ET AL.: "Allosteric regulators of reversible hemoglobin oxygenation" BIOORG. KHIM., vol. 16, no. 9, 1990, pages 1157-1176, XP002156415 page 1176	1-49
Y	J.-P- VIGNERON ET AL.: "Guanidinium-cholesterol cationic lipids: Efficient vectors for the transfection of eukaryotic cells" PROC. NATL. ACAD. SCI. USA, vol. 93, no. 18, 1996, pages 9682-9686, XP002030510 * entire document *	1-49
Y	N. OUDRHIRI ET AL.: "Gene transfer by guanidinium-cholesterol cationic lipids into airway epithelial cells in vitro and in vivo" PROC. NATL. ACAD. SCI. USA, vol. 94, no. 5, 1997, pages 1651-1656, XP002035783 * entire document *	1-49
Y	B. PITARD ET AL.: "Structural characteristics of supramolecular assemblies formed by guanidinium-cholesterol reagents for gene transfection" PROC. NATL. ACAD. SCI. USA, vol. 96, no. 6, 1999, pages 2621-2626, XP000941540 * entire documents *	1-49
Y	US 5 599 974 A (D. J. ABRAHAM ET AL.) 4 February 1997 (1997-02-04) tables 1,2	1-49
Y	WO 92 20335 A (CENTER FOR INNOVATIVE TECHNOLOGY) 26 November 1992 (1992-11-26) claims 1-12	1-49

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 731 454 A (D. J. ABRAHAM ET AL.) 24 March 1998 (1998-03-24) column 29, line 1 -column 29, line 29 ---	1-49
Y	US 5 432 191 A (D. J. ABRAHAM ET AL.) 11 July 1995 (1995-07-11) column 21, line 23 -column 22, line 5; table 4 ---	1-49
Y	J.-P. VIGNERON: "Supramolecular Bioorganic Chemistry: Nucleic Acids Recognition and Synthetic Vectors for Gene Transfer" MOLECULES, vol. 4, 1999, pages 180-203, XP002156416 * entire document * -----	1-49

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/22583

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 338916 A	25-10-1989	FR 2630329 A	27-10-1989
		AT 77753 T	15-07-1992
		DE 68901944 D	06-08-1992
		DE 68901944 T	10-12-1992
		ES 2043054 T	16-12-1993
		GR 3005220 T	24-05-1993
		JP 2042023 A	13-02-1990
		US 5110909 A	05-05-1992
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WO 9839358 A	11-09-1998	US 5877220 A	02-03-1999
		AU 6687998 A	22-09-1998
WO 9839359 A	11-09-1998	US 6034135 A	07-03-2000
		AU 6448898 A	22-09-1998
		EP 0968227 A	05-01-2000
EP 452055 A	16-10-1991	US 5114768 A	19-05-1992
		DE 69106562 D	23-02-1995
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US 5612207 A	18-03-1997	AU 680890 B	14-08-1997
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		US 5731454 A	24-03-1998
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		US 5872282 A	16-02-1999
		US 5591892 A	07-01-1997
		US 5648375 A	15-07-1997
		US 5705521 A	06-01-1998
		US 5661182 A	26-08-1997
		CA 2109575 A	26-11-1992
		EP 0585366 A	09-03-1994
		JP 3023423 B	21-03-2000
		JP 7508973 T	05-10-1995
		US 5250701 A	05-10-1993
		WO 9220335 A	26-11-1992
		US 5248785 A	28-09-1993
		CA 2051693 A	13-08-1991
		DE 69115790 D	08-02-1996
		DE 69115790 T	23-05-1996
		EP 0471811 A	26-02-1992
		JP 3023422 B	21-03-2000
		JP 4506812 T	26-11-1992
		WO 9112235 A	22-08-1991
US 5872282 A	16-02-1999	US 5731454 A	24-03-1998
		US 5432191 A	11-07-1995

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/22583

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5872282 A		US 5382680 A	17-01-1995
		US 5591892 A	07-01-1997
		US 5677330 A	14-10-1997
		US 5648375 A	15-07-1997
		US 5705521 A	06-01-1998
		US 5927283 A	27-07-1999
		US 5661182 A	26-08-1997
		US 5250701 A	05-10-1993
US 5110909 A	05-05-1992	FR 2630329 A	27-10-1989
		AT 77753 T	15-07-1992
		DE 68901944 D	06-08-1992
		DE 68901944 T	10-12-1992
		EP 0338916 A	25-10-1989
		ES 2043054 T	16-12-1993
		GR 3005220 T	24-05-1993
		JP 2042023 A	13-02-1990
US 4921997 A	01-05-1990	EP 0420930 A	10-04-1991
		JP 3505728 T	12-12-1991
		WO 8912622 A	28-12-1989
		US 5472981 A	05-12-1995
		US 5093367 A	03-03-1992
		US 5292935 A	08-03-1994
US 5079337 A	07-01-1992	FR 2600894 A	08-01-1988
		AT 67673 T	15-10-1991
		CA 1328602 A	19-04-1994
		DE 3773309 A	31-10-1991
		EP 0259197 A	09-03-1988
		ES 2026190 T	16-04-1992
		WO 8800055 A	14-01-1988
		GR 3003240 T	17-02-1993
		JP 1500524 T	23-02-1989
JP 04300838 A	23-10-1992	NONE	
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WO 9220335 A	26-11-1992	US 5122539 A	16-06-1992
		US 5248785 A	28-09-1993
		CA 2109575 A	26-11-1992
		EP 0585366 A	09-03-1994
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		US 5290803 A	01-03-1994
		US 5250701 A	05-10-1993
		US 5432191 A	11-07-1995
		US 5731454 A	24-03-1998
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/22583

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[Continued on next page]

(54) Title: ENHANCED OXYGEN DELIVERY IN MAMMALS, METHODS AND REAGENTS RELATED THERETO

Summary of Certain Experiments Forming IHP-BGTC Complexes

0.35 mM BGTC suspension (spin, sonicated, 50 C)
188 nm particles (measured by light scattering)
Two populations: i) 56%, 116 nm; ii) 51%, 558 nm

1 mM IHP
clear solution

1 mM IHP + 0.35 mM BGTC
(i) precipitation (760 nm-980 nm-1200 nm-1706 nm-2000 nm-2800 nm) measurement stopped after five minutes
(ii) sonicated back to 760 nm, but then particle size increased to 2000 nm
(iii) 10 µL of serum added, but the size of the particles did not change; subsequent sonication had no discernable effect

2 mL HBSE + 100 µL serum particle size = 930 nm
(i) addition of BGTC (0.35 mM final concentration): precipitation
(ii) addition of IHP (1.0 mM final concentration): precipitation, but no greater than without IHP

BGTC at 0.35 mM, 3.5 mM, or 35 mM, each with 1 mM IHP
precipitation (particle size = 800 nm), but 3% DMF limited the particle size to about 480 nm

IHP at 1 mM, 2 mM, 5 mM, or 10 mM, with 0.35 mM or 3.5 mM BGTC
precipitation, but 3% DMF limited the particle size

Concentrations described in (6), including DMF; Tris pH 7.1; and washed RBCs, lysed cells, or hemoglobin: precipitation

(57) Abstract: The present invention comprises compounds, compositions thereof, and methods capable of delivering a broad range of anionic molecules to the cytoplasm of mammalian cells. In certain embodiments, the present invention relates to compounds, compositions thereof, and methods that enhance the ability of mammalian red blood cells to deliver oxygen, by delivering a ligand for the allosteric site of hemoglobin to the cytoplasm of the blood cells.

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(54) Title: AGENTS FOR THE ENHANCED OXYGEN DELIVERY IN MAMMALS

(57) Abstract: The present invention comprises compounds, compositions thereof, and methods capable of delivering a broad range of anionic molecules to the cytoplasm of mammalian cells. In certain embodiments, the present invention relates to compounds, compositions thereof, and methods that enhance the ability of mammalian red blood cells to deliver oxygen, by delivering a ligand for the allosteric site of hemoglobin to the cytoplasm of the blood cells.

AGENTS FOR THE ENHANCED OXYGEN DELIVERY IN MAMMALS

Related Applications

This application claims the benefit of priority to United States Provisional Patent Application serial number 60/150,574, filed August 25, 1999.

Background of the Invention

Ischemia

Ischemic insult, i.e., the localized deficiency of oxygen to an organ or skeletal tissue, is a common and important problem in many clinical conditions. The problem is especially acute in organ transplant operations in which a harvested organ is removed from a body, isolated from a blood source, and thereby deprived of oxygen and nutrients for an extended period of time. Ischemic insult also occurs in certain clinical conditions, such as sickle cell anemia and septic shock, which may result from hypotension or organ dysfunction. Depending on the duration of the insult, the ischemia can disturb cellular metabolism and ion gradients, and ultimately cause irreversible cellular injury and death.

Ischemia is also associated with various clinical conditions, such as septic shock. Septic shock as a result of hypotension and organ dysfunction in response to infectious sepsis is a major cause of death. The manifestations of sepsis include those related to the systemic response to infection (tachycardia, tachypnea alterations in temperature and leukocytosis) and those related to organ-system dysfunction (cardiovascular, respiratory, renal, hepatic and hematologic abnormalities). Furthermore, the lipopolysaccharide (LPS) of gram-negative bacteria is considered to be the most important exogenous mediator of acute inflammatory response to septic shock. The LPS or endotoxin released from the outer membrane of gram-negative bacteria results in the release of cytokines and other cellular mediators, including tumor necrosis factor alpha (TNF alpha), interleukin-1 (Il-1), interleukin-6 (Il-6) and thromboxane A2. Extreme levels of these mediators are known to trigger many pathological events, including fever, shock, and intravascular coagulation, leading to ischemia and organ failure.

Sickle cell anemia is another condition associated with ischemia. Sickle cell anemia is a classical phenotype of hereditary hemoglobinopathy with hemoglobin S instead of normal hemoglobin A. Sickle cell anemia is associated with hypoxia because of decreased oxygen tension with hemoglobin S. This condition leads to a systemic hypoxic condition. The viscosity of deoxygenated blood is related to the proportion of sickled red cells, capillary stasis and pain

crisis.

Arguably, heart attacks and stroke are the most widely recognized example of the damage resulting from ischemia. Myocardial ischemia is a condition wherein there is insufficient blood supply to the myocardium (the muscles of the heart) to meet its demand for oxygen. The ultimate result of persistent myocardial ischemia is necrosis or death of a portion of cardiac muscle tissue, known as a myocardial infarct, commonly known as a heart attack.

Insufficient blood supply to the myocardium is generally due to an obstruction or thrombus in an artery supplying blood to the myocardium. Another cause can be atrial fibrillation, wherein the increased heart rate associated with atrial fibrillation increases the work, and hence the blood demand of the myocardium, while the atrial fibrillation at the same time reduces the blood supply.

Whereas stroke is defined as a sudden impairment of body functions caused by a disruption in the supply of blood to the brain. For instance, a stroke occurs when blood supply to the brain is interrupted for any reason, including hemorrhage, low blood pressure, clogging by atherosclerotic plaque, a blood clot, or any particle. Because of the blockage or rupture, part of the brain fails to get the supply of blood and oxygen that it requires. Brain tissue that receives an inadequate supply of blood is said to be ischemic. Deprived of oxygen and nutrients, nerve cells and other cell types within the brain begin to fail, creating an infarct (an area of cell death, or necrosis). As the neurons fail and die, the part of the body controlled by those neurons can no longer function. The devastating effects of ischemia are often permanent because brain tissue has very limited repair capabilities and lost neurons are typically not regenerated.

Cerebral ischemia may be incomplete (blood flow is reduced but not entirely cut off), complete (total loss of tissue perfusion), transient or permanent. If ischemia is incomplete and persists for no more than ten to fifteen minutes, neural death may not occur. More prolonged or complete ischemia results in infarction. Depending on the site and extent of the infarction, mild to severe neurological disability or death will follow.

To a modest extent, the brain is protected against cerebral ischemia by compensatory mechanisms, including collateral circulation (overlapping local blood supplies), and arteriolar auto-regulation (local smooth muscle control of blood flow in the smallest arterial channels). If compensatory mechanisms operate efficiently, slightly diminished cerebral blood flow produces neither tissue ischemia nor abnormal signs and symptoms. Usually, such mechanisms must act within minutes to restore blood flow if permanent infarction damage is to be avoided or reduced. Arteriolar auto-regulation works by shunting blood from noncritical regions to infarct zones.

Even in the face of systemic hypotension, auto-regulation may be sufficient to adjust the

circulation and thereby preserve the vitality and function of brain or heart tissue. Alternatively, ischemia may be sufficiently prolonged and compensatory mechanisms sufficiently inadequate that a catastrophic stroke or heart attack results.

Hemoglobin

Hemoglobin is a tetrameric protein which delivers oxygen via an allosteric mechanism. Oxygen binds to the four hemes of the hemoglobin molecule. Each heme contains porphyrin and iron in the ferrous state. The ferrous iron-oxygen bond is readily reversible. Binding of the first oxygen to a heme releases much greater energy than binding of the second oxygen molecule, binding of the third oxygen releases even less energy, and binding of the fourth oxygen releases the least energy.

In blood, hemoglobin is in equilibrium between two allosteric structures. In the "T" (for tense) state, hemoglobin is deoxygenated. In the "R" (for relaxed) state, hemoglobin is oxygenated. An oxygen equilibrium curve can be scanned to observe the affinity and degree of cooperativity (allosteric action) of hemoglobin. In the scan, the Y-axis plots the percent of hemoglobin oxygenation and the X-axis plots the partial pressure of oxygen in millimeters of mercury (mm Hg). If a horizontal line is drawn from the 50% oxygen saturation point to the scanned curve and a vertical line is drawn from the intersection point of the horizontal line with the curve to the partial pressure X-axis, a value commonly known as the P50 is determined (i.e., this is the pressure in mm Hg when the scanned hemoglobin sample is 50% saturated with oxygen). Under physiological conditions (i.e., 37 C, pH = 7.4, and partial carbon dioxide pressure of 40 mm Hg), the P50 value for normal adult hemoglobin (HbA) is around 26.5 mm Hg. If a lower than normal P50 value is obtained for the hemoglobin being tested, the scanned curve is considered to be "left-shifted" and the presence of high oxygen-affinity hemoglobin is indicated. Conversely, if a higher than normal P50 value is obtained for the hemoglobin being tested, the scanned curve is considered to be "right-shifted", indicating the presence of low oxygen-affinity hemoglobin.

It has been proposed that influencing the allosteric equilibrium of hemoglobin is a viable avenue of attack for treating diseases. The conversion of hemoglobin to a high affinity state is generally regarded to be beneficial in resolving problems with (deoxy)hemoglobin-S (i.e., sickle cell anemia). The conversion of hemoglobin to a low affinity state is believed to have general utility in a variety of disease states where tissues suffer from low oxygen tension, such as ischemia and radio sensitization of tumors. Several synthetic compounds have been identified which have utility in the allosteric regulation of hemoglobin and other proteins. For example,

several new compounds and methods for treating sickle cell anemia which involve the allosteric regulation of hemoglobin are reported in U.S. Pat. No. 4,699,926 to Abraham et al., U.S. Pat. No. 4,731,381 to Abraham et al., U.S. Pat. No. 4,731,473 to Abraham et al., U.S. Pat. No. 4,751,244 to Abraham et al., and U.S. Pat. No. 4,887,995 to Abraham et al. Furthermore, in both Perutz, "Mechanisms of Cooperativity and allosteric Regulation in Proteins", Quarterly Reviews of Biophysics 22, 2 (1989), pp. 163-164, and Lalezari et al., "LR16, a compound with potent effects on the oxygen affinity of hemoglobin, on blood cholesterol, and on low density lipoprotein", Proc. Natl. Acad. Sci., USA 85 (1988), pp. 6117-6121, compounds which are effective allosteric hemoglobin modifiers are discussed. In addition, Perutz et al. has shown that a known antihyperlipoproteinemia drug, bezafibrate, is capable of lowering the affinity of hemoglobin for oxygen (See "Bezafibrate lowers oxygen affinity of hemoglobin", Lancet 1983, 881).

Human normal adult hemoglobin ("HbA") is a tetrameric protein containing two alpha chains having 141 amino acid residues each and two beta chains having 146 amino acid residues each, and also bearing prosthetic groups known as hemes. The erythrocytes help maintain hemoglobin in its reduced, functional form. The heme-iron atom is susceptible to oxidation, but may be reduced again by one of two systems within the erythrocyte, the cytochrome b5, and glutathione reduction systems.

Hemoglobin is able to alter its oxygen affinity, thereby increasing the efficiency of oxygen transport in the body due to its dependence on 2,3-DPG, an allosteric regulator. 2,3-DPG is present within erythrocytes at a concentration that facilitates hemoglobin to release bound oxygen to tissues. Naturally-occurring hemoglobin includes any hemoglobin identical to hemoglobin naturally existing within a cell. Naturally-occurring hemoglobin is predominantly wild-type hemoglobin, but also includes naturally-occurring mutant hemoglobin. Wild-type hemoglobin is hemoglobin most commonly found within natural cells. Wild-type human hemoglobin includes hemoglobin A, the normal adult human hemoglobin having two alpha - and two beta-globin chains. Mutant hemoglobin has an amino-acid sequence that differs from the amino-acid sequence of wild-type hemoglobin as a result of a mutation, such as a substitution, addition or deletion of at least one amino acid. Adult human mutant hemoglobin has an amino-acid sequence that differs from the amino-acid sequence of hemoglobin A. Naturally-occurring mutant hemoglobin has an amino-acid sequence that has not been modified by humans. The naturally-occurring hemoglobin of the present invention is not limited by the methods by which it is produced. Such methods typically include, for example, erythrocytolysis and purification, recombinant production, and protein synthesis.

It is known that hemoglobin specifically binds small polyanionic molecules, especially

2,3-diphosphoglycerate (DPG) and adenosine triphosphate (ATP), present in the mammalian red cell (Benesch and Benesch, Nature, Vol. 221, p. 618, 1969). This binding site is located at the centre of the tetrameric structure of hemoglobin (Arnone, A., Nature, Vol. 237, p. 146, 1972). The binding of these polyanionic molecules is important in regulating the oxygen-binding affinity of hemoglobin since it allosterically affects the conformation of hemoglobin leading to a decrease in oxygen affinity (Benesch and Benesch, Biochem. Biophys. Res. Comm., Vol. 26, p. 162, 1967). Conversely, the binding of oxygen allosterically reduces the affinity of hemoglobin for the polyanion. (Oxy) hemoglobin therefore binds DPG and ATP weakly. This is shown, for example, by studies of spin-labelled ATP binding to oxy- and deoxyhemoglobin as described by Ogata and McConnell (Ann. N.Y. Acad. Sc., Vol. 222, p. 56, 1973). In order to exploit the polyanion-binding specificity of hemoglobin, or indeed to perform any adjustment of its oxygen-binding affinity by chemically modifying the polyanion binding site, it has been necessary in the prior art that hemoglobin be deoxygenated. However, hemoglobin as it exists in solutions, or mixtures exposed to air, is in its oxy state, i.e., (oxy)hemoglobin. In fact it is difficult to maintain hemoglobin solutions in the deoxy state, (deoxy)hemoglobin, throughout a chromatographic procedure. Because of these difficulties, the technique of affinity chromatography has not been used in the prior art to purify hemoglobin.

Hemoglobin has also been administered as a pretreatment to patients receiving chemotherapeutic agents or radiation for the treatment of tumors (U.S. Pat. No. 5,428,007; WO 92/20368; WO 92/20369), for prophylaxis or treatment of systemic hypotension or septic shock induced by internal nitric oxide production (U.S. Pat. No. 5,296,466), during the perioperative period or during surgery in a method for maintaining a steady-state hemoglobin concentration in a patient (WO 95/03068), and as part of a perioperative hemodilution procedure used prior to surgery in an autologous blood use method (U.S. Pat. Nos. 5,344,393 and 5,451,205). When a patient suffers a trauma (i.e., a wound or injury) resulting, for example, from surgery, an invasive medical procedure, or an accident, the trauma disturbs the patient's homeostasis. The patient's body biologically reacts to the trauma to restore homeostasis. This reaction is referred to herein as a naturally occurring stress response. If the body's stress response is inadequate or if it occurs well after the trauma is suffered, the patient is more prone to develop disorders.

Reduction of the Oxygen-Affinity of Hemoglobin

The major function of erythrocytes consists in the transport of molecular oxygen from the lungs to the peripheral tissues. The erythrocytes contain a high concentration of hemoglobin (30 pg per cell=35.5 g/100 ml cells) which forms a reversible adduct with O_2. The O_2-partial

pressure in the lung is about.100 mm Hg, in the capillary system is about.70 mm Hg, against which O_2 must be dissociated from the oxygenated hemoglobin. Under physiological conditions, only about 25% of the oxygenated hemoglobin may be deoxygenated; about.75% is carried back to the lungs with the venous blood. Thus, the major fraction of the hemoglobin-O_2 adduct is not used for the O_2 transport.

Interactions of hemoglobin with allosteric effectors enable an adaptation to the physiological requirement of maximum O_2 release from the hemoglobin-O_2 adduct with simultaneous conservation of the highest possible O_2 partial pressure in the capillary system. 2,3-Diphosphoglycerate increases the half-saturation pressure of stripped hemoglobin at pH 7.4 from $P(O_2)$ (1/2)=9.3 mm Hg (37 C), and 4.3 mm Hg (25 C) to $P(O_2)$ (1/2)=23.7 mm Hg (37 C), and 12.0 mm Hg (25 C), respectively (Imai, K. and Yonetani, T. (1975), J. Biol. Chem. 250, 1093-1098). A significantly stronger decrease of the O_2 affinity, i.e., enhancement of the O_2 half-saturation pressure has been achieved for stripped hemoglobin by binding of inositol hexaphosphate (phytic acid; IHP) (Ruckpaul, K. et al. (1971) Biochim. Biophys. Acta 236, 211-221) isolated from vegetal tissues. Binding of IHP to hemoglobin increases the O_2 half-saturation pressure to $P(O_2)$ (1/2)=96.4 mm Hg (37 C.), and $P(O_2)$ (1/2)=48.4 mm Hg (25 C.), respectively. IHP, like 2,3-diphosphoglycerate and other polyphosphates cannot penetrate the erythrocyte membrane.

Furthermore, the depletion of DPG and ATP in stored red cells leads to a progressive increase of the oxygen affinity of hemoglobin contained therein (Balcerzak, S. et al. (1972) Adv. Exp. Med. Biol. 28, 453-447). The O_2-binding isotherms are measured in the absence of CO_2 and at constant pH (pH 7.4) in order to preclude influences of these allosteric effectors on the half-saturation pressure. The end point of the progressive polyphosphate depletion is defined by $P(O_2)$ (1/2)=4.2 mm Hg, which is the half-saturation pressure of totally phosphate-free (stripped) hemoglobin; the starting point, i.e., $P(O_2)$ (1/2) of fresh erythrocytes, depends on the composition of the suspending medium. From these polyphosphate depletion curves a new functional parameter of stored erythrocytes can be determined, the so-called half-life time of intra-erythrocytic polyphosphate: 9 d (days) in isotonic 0.1 M bis-Tris buffer pH 7.4; and 12 d (days) in acid-citrate-dextrose conservation (ACD) solution.

Several years ago, it was discovered that the antilipidemic drug clofibric acid lowered the oxygen affinity of hemoglobin solutions (Abraham et al., J. Med. Chem. 25, 1015 (1982), and Abraham et al., Proc. Natl. Acad. Sci. USA 80, 324 (1983)). Bezafibrate, another antilipidemic drug, was later found to be much more effective in lowering the oxygen affinity of hemoglobin solutions and suspensions of fresh, intact red cells (Perutz et al., Lancet, 881, Oct. 15, 1983).

Subsequently, X-ray crystallographic studies have demonstrated that clofibric acid and bezafibrate bind to the same sites in the central water cavity of deoxyhemoglobin, and that one bezafibrate molecule will span the sites occupied by two clofibric acid molecules. Bezafibrate and clofibric acid act by stabilizing the deoxy structure of hemoglobin, shifting the allosteric equilibrium toward the low affinity deoxy form. Bezafibrate and clofibric acid do not bind in any specific manner to either oxy- or carbonmonoxyhemoglobin.

In more recent investigations, a series of urea derivatives [2-[4-[[(arylamino)carbonyl]amino]phenoxy]-2-methylpropionic acids] was discovered that has greater allosteric potency than bezafibrate at stabilizing the deoxy structure of hemoglobin and shifting the allosteric equilibrium toward the low oxygen affinity form (Lalezari, Proc. Natl. Acad. Sci. USA 85, 6117 (1988)).

Drugs which can allosterically modify hemoglobin toward a lower oxygen affinity state hold potential for many clinical applications, such as for the treatment of ischemia, shock, and polycythemia, and as radiosensitizing agents. Unfortunately, the effects of bezafibrate and the urea derivatives discussed above have been found to be significantly inhibited by serum albumin, the major protein in blood serum (Lalezari et al., Biochemistry, 29, 1515 (1990)). Therefore, the clinical usefulness of these drugs is seriously undermined because in whole blood and in the body, the drugs would be bound by serum albumin instead of reaching the red cells, crossing the red cell membrane, and interacting with hemoglobin protein molecule to produce the desired effect.

There has been considerable interest in medicine, the military health services, and the pharmaceutical industry in finding methods to increase oxygen delivery in vivo for ischemic insults, stroke, and trauma; to increase blood storage life; to discover radio sensitization agents; and to develop new blood substitutes. In all these instances, the availability of either autologous blood or recombinant Hb solutions is of major interest, provided the oxygen affinity can be decreased to enhance oxygen delivery to the tissues.

2,3-Diphosphoglycerate (2,3-DPG) is the normal physiological ligand for the allosteric site on hemoglobin. However, phosphorylated inositols are found in the erythrocytes of birds and reptiles. Specifically, inositol hexaphosphate (IHP), as known as phytic acid, displaces hemoglobin-bound 2,3-DPG, binding to the allosteric site with one-thousand times greater affinity. Unfortunately, IHP is unable to pass unassisted across the erythrocyte membrane.

Enhanced Oxygen Delivery in Mammals

The therapy of oxygen deficiencies requires the knowledge of parameters which characterize both the O_2 transport capacity and the O_2 release capacity of human RBCs. The

parameters of the O_2 transport capacity, i.e., Hb concentration, the number of RBCs, and hemocrit, are commonly used in clinical diagnosis. However, the equally important parameters of the O_2 release capacity, i.e., O_2 half-saturation pressure of Hb and RBCs, and the amounts of high and low oxygen affinity hemoglobins in RBCs, are not routinely determined and were not given serious consideration until pioneering work by Gerosonde and Nicolau (*Blut*, **1979**, *39*, 1-7).

In the 1980s, Nicolau et al. (J. Appl. Physiol. 58:1810-1817 (1985); PHYTIC ACID: Chemsitry and Applications; Graf, E., Ed.; Pilatus Press, Minneapolis, MN, USA; 1986; and *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 6894-6898) reported that the encapsulation in red blood cells (RBCs) of IHP, via a technique of controlled lysis and resealing, results in a significant decrease in the hemoglobin affinity for oxygen. The procedure yielded RBCs with unchanged life spans, normal ATP and K+ levels, and normal rheological competence. Enhancement of the O_2-release capacity of these cells brought about significant physiological effects in piglets: 1) reduced cardiac output, linearly dependent on the P50 value of the RBCs; 2) increased arteriovenous difference; and 3) improved tissue oxygenation. Long term experiments showed that in piglets the high P50 value of IHP-RBCs was maintained over the entire life spans of the RBCs.

More recently, Nicolau et al. (*TRANSFUSION* **1995**, *35*, 478-486; and US Patent 5,612,207) reported the use of a large-volume, continuous-flow electroporation system for the encapsulating IHP in human RBCs. These modified RBCs possess P50 values of approximately 50 torr, roughly twice that of unmodified human RBCs. Additionally, 85% of the RBCs survived the electroporation process, displaying hematologic indices nearly identical to those of unmodified RBCs. Nicolau's electroporation system processes one unit of blood every ninety minutes.

Specific Clinical Applications of Enhanced Oxygen Delivery

There are numerous clinical conditions that would benefit from treatments that would increase tissue delivery of oxygen bound to hemoglobin. For example, the leading cause of death in the United States today is cardiovascular disease. The acute symptoms and pathology of many cardiovascular diseases, including congestive heart failure, myocardial infarction, stroke, intermittent claudication, and sickle cell anemia, result from an insufficient supply of oxygen in fluids that bathe the tissues. Likewise, the acute loss of blood following hemorrhage, traumatic injury, or surgery results in decreased oxygen supply to vital organs. Without oxygen, tissues at sites distal to the heart, and even the heart itself, cannot produce enough energy to sustain their

normal functions. The result of oxygen deprivation is tissue death and organ failure.

Although the attention of the American public has long been focused on the preventive measures required to alleviate heart disease, such as exercise, appropriate dietary habits, and moderation in alcohol consumption, deaths continue to occur at an alarming rate. Since death results from oxygen deprivation, which in turn results in tissue destruction and/or organ dysfunction, one approach to alleviate the life-threatening consequences of cardiovascular disease is to increase oxygenation of tissues during acute stress. The same approach is also appropriate for persons suffering from blood loss or chronic hypoxic disorders, such as congestive heart failure.

Another condition which could benefit from an increase in the delivery of oxygen to the tissues is anemia. A significant portion of hospital patients experience anemia or a low "crit" caused by an insufficient quantity of red blood cells or hemoglobin in their blood. This leads to inadequate oxygenation of their tissues and subsequent complications. Typically, a physician can temporarily correct this condition by transfusing the patient with units of packed red blood cells.

Enhanced blood oxygenation may also reduce the number of heterologous transfusions and allow use of autologous transfusions in more case. The current method for treatment of anemia or replacement of blood loss is transfusion of whole human blood. It is estimated that three to four million patients receive transfusions in the U.S. each year for surgical or medical needs. In situations where there is more time it is advantageous to completely avoid the use of donor or heterologous blood and instead use autologous blood.

Often the amount of blood which can be drawn and stored prior to surgery limits the use of autologous blood. Typically, a surgical patient does not have enough time to donate a sufficient quantity of blood prior to surgery. A surgeon would like to have several units of blood available. As each unit requires a period of several weeks between donations and can not be done less than two weeks prior to surgery, it is often impossible to sequester an adequate supply of blood. By processing autologous blood with IHP, less blood is required and it becomes possible to completely avoid the transfusion of heterologous blood.

Because IHP-treated RBCs may release up to 2-3 times as much oxygen as untreated red cells, in many cases, a physician will need to transfuse fewer units of IHP-treaded red cells. This exposes the patient to less heterologous blood, decreases the extent of exposure to vital diseases from blood donors and minimizes immune function disturbances secondary to transfusions. The ability to infuse more efficient red blood cells is also advantageous when the patients blood

volume is excessive. In more severe cases, where oxygen transport is failing, the ability to improve rapidly a patient's tissue oxygenation is life saving.

Although it is evident that methods of enhancing oxygen delivery to tissues have potential medical applications, currently there are no methods clinically available for increasing tissue delivery of oxygen bound to hemoglobin. Transient, 6 to 12 hour elevations of oxygen deposition have been described in experimental animals using either DPG or molecules that are precursors of DPG. The natural regulation of DPG synthesis in vivo and its relatively short biological half-life, however, limit the DPG concentration and the duration of increased tissue PO_2, and thus limit its therapeutic usefulness.

Additionally, as reported in Genetic Engineering News, Vol. 12, No. 6, Apr. 15, 1992, several groups are attempting to engineer free oxygen-carrying hemoglobin as a replacement for human blood. Recombinant, genetically modified human hemoglobin that does not break down in the body and that can readily release up to 30% of its bound oxygen is currently being tested by Somatogen, Inc., of Boulder Colo. While this product could be useful as a replacement for blood lost in traumatic injury or surgery, it would not be effective to increase PO_2 levels in ischemic tissue, since its oxygen release capacity is equivalent to that of natural hemoglobin (27-30%). As are all recombinant products, this synthetic hemoglobin is also likely to be a costly therapeutic.

Synthetic human hemoglobin has also been produced in neonatal pigs by injection of human genes that control hemoglobin production. This product may be less expensive product than the Somatogen synthetic hemoglobin, but it does not solve problems with oxygen affinity and breakdown of hemoglobin in the body.

Summary of the Invention

The present invention relates to compositions, and methods of use thereof, consisting essentially of a cationic, lipophilic, water-soluble molecule (e.g., a bis-guanidinium cholesterol), and an anionic ligand for the allosteric site of hemoglobin, e.g., inositol hexaphosphate (IHP). In certain embodiments, the present invention is related to compounds and compositions thereof which deliver into erythrocytes allosteric modifiers of hemoglobin *in vivo*. Additionally, the invention is directed to the use of the compounds or compositions thereof that are effective in delivering into erythrocytes allosteric modifiers of hemoglobin, lowering the oxygen affinity state in red blood cell suspensions and whole blood. It is an object of this invention to provides methods for delivering into erythrocytes allosteric modifiers of hemoglobin in whole blood and

in vivo, utilizing compounds or compositions thereof that do not lose their effectiveness in the presence of normal concentrations of the remaining components of whole blood.

Ligands for the allosteric site of hemoglobin interact with the hemoglobin molecule and impact its ability to bind oxygen. This invention is particularly concerned with the delivery into erythrocytes of ligands for the hemoglobin allosteric site, causing oxygen to be bound relatively less tightly to hemoglobin, such that oxygen is off-loaded from the hemoglobin molecule more easily.

The process of allosterically modifying hemoglobin towards a lower oxygen affinity state in whole blood and in vivo may be used in a wide variety of applications including treatments for ischemia, heart disease, wound healing, radiation therapy of cancer, and adult respiratory distress syndrome (ARDS). Furthermore, a decrease in the oxygen affinity of hemoglobin in whole blood will extend its shelf-life, or restore the oxygen carrying capacity of aged blood.

Brief Description of the Figures

Figure 1 presents a summary of certain experiments forming inositol hexaphosphate-bisguanidinium cholesterol (IHP-BGTC) complexes.

Figure 2 depicts an Hb-O_2 dissociation curve in human RBCs after incubation with the IHP-BGTC system for 60 min. at room temperature [C_a = controls incubated with IHP (1 mM)-DMF (3%); 1a = RBCs + IHP (1 mM)-BGTC (0.35 mM)-DMF (3%); 2a = RBCs + IHP (1 mM)-BGTC (3.5 mmol)-DMF (3%); 3a = IHP (2 mM)-BGTC (0.35 mM)-DMF (3%)].

Detailed Description of the Invention

The process of allosterically modifying hemoglobin towards a low oxygen affinity state in whole blood and in vivo could be used in a wide variety of applications including in treatments for ischemia, heart disease, wound healing, radiation therapy of cancer, adult respiratory distress syndrome (ARDS), etc., in extending the shelf-life of blood or restoring the oxygen carrying capacity of out-dated blood, and as sensitizers for x-ray irradiation in cancer therapy, as well as in many other applications.

This invention is related to the use of allosteric hemoglobin modifier compounds in red blood cell suspensions, in whole blood, and in vivo. Serum albumin, which is the most abundant protein in blood plasma, has been identified as inhibiting the allosteric effects of clofibric acid, bezafibrate, and L3,5/L3,4,5. The precise nature of this inhibition is not fully understood, but appears to be related to these compounds binding to the serum albumin. By contrast, as will be discussed in detail below, the RSR compounds have been found to be relatively unaffected by the

presence of serum albumin. Ligands for the allosteric site of hemoglobin that are not adversely effected by serum albumin represent particularly good candidates for drug applications, since the performance of the drug will not be frustrated by the presence of serum albumin present in a patient's blood.

This invention relates to the incorporation of a wide variety of therapeutically useful substances into mammalian red blood cells (RBCs), which could not previously be accomplished without unacceptable losses of RBC contents and/or integrity. More particularly, the compounds and methods of the present invention makes possible the introduction or incorporation of anionic agents into RBCs, such as DNA, RNA, chemotherapeutic agents, and antibiotic agents. These and other water soluble substances may be used for a desired slow continuous delivery or targeted delivery when the treated RBC carrier is later injected in vivo. The particular polyanion to be selected can be based on whether an allosteric effector of hemoglobin would be desirable for a particular treatment.

The present invention provides a novel method for increasing the oxygen-carrying capacity of erythrocytes. In accordance with the method of the present invention, the IHP combines with hemoglobin in a stable way, and shifts its oxygen releasing capacity. Erythrocytes with IHP-hemoglobin can release more oxygen per molecule than hemoglobin alone, and thus more oxygen is available to diffuse into tissues for each unit of blood that circulates. Injected in vivo, IHP is toxic and cannot be tolerated as an ordinary drug.

Another advantage of IHP-treated red blood cells is that they show the Bohr effect in circulation and when stored. Normal red blood cells that have been stored do not regain their maximum oxygen carrying capacity in circulation for approximately 24 hours. This is because the DPG present in normal red blood cells is degraded by native enzymes, e.g., phosphatases, during storage and must be replaced by the body after transfusion. In contrast, red blood cells treated according to the present invention retain their maximum oxygen carrying capacity during storage and therefore can deliver oxygen to the tissues in response to demand immediately after transfusion into a human or animal because there are no native enzymes in erythrocytes which degrade IHP.

IHP-treated RBCs may be used in the treatment of acute and chronic conditions, including, but not limited to, hospitalized patients, cardiovascular operations, chronic anemia, anemia following major surgery, coronary infarction and associated problems, chronic pulmonary disease, cardiovascular patients, autologous transfusions, as an enhancement to packed red blood cells transfusion (hemorrhage, traumatic injury, or surgery). congestive heart failure, myocardial infarction (heart attack), stroke, peripheral vascular disease, intermittent

claudication, circulatory shock, hemorrhagic shock, anemia and chronic hypoxia, respiratory alkalemia, metabolic alkalosis, sickle cell anemia, reduced lung capacity caused by pneumonia, surgery, pneumonia, trauma, chest puncture, gangrene, anaerobic infections, blood vessel diseases such as diabetes, substitute or complement to treatment with hyperbaric pressure chambers, intra-operative red cell salvage, cardiac inadequacy, anoxia-secondary to chronic indication, organ transplant, carbon monoxide, nitric oxide, and cyanide poisoning.

Treating a human or animal for any one or more of the above disease states is done by transfusing into the human or animal between approximately 0.1 and 6 units (1 unit = 500 mL) of IHP-treated blood that has been prepared according to the present invention. In certain cases, blood exchange with IHP-treated blood may be possible. The volume of IHP-treated red blood cells that is administered to the human or animal will depend upon the value of P50 for the IHP-treated RBCs. It is to be understood that the volume of IHP-treated red blood cells that is administered to the patient can vary and still be effective. IHP-treated RBCs are similar to normal red blood cells in every respect except that their P50 value is shifted towards higher partial pressures of O_2. Erythrocytes release oxygen only in response to demand by organs and tissue. Therefore, the compounds, compositions thereof, and methods of the present invention will only restore a normal level of oxygenation to healthy tissue, avoiding the cellular damage that is associated with an over-abundance of oxygen.

Because the compounds, compositions, and methods of the present invention are capable of allosterically modifying hemoglobin to favor the low oxygen affinity "T" state (i.e., right shifting the equilibrium curve), they will be useful in treating a variety of disease states in mammals, including humans, wherein tissues suffer from low oxygen tension, such as cancer and ischemia. Furthermore, as disclosed by Hirst et al. (Radiat. Res., Vol. 112, (1987), pp. 164), decreasing the oxygen affinity of hemoglobin in circulating blood has been shown to be beneficial in the radiotherapy of tumors. The compounds and compositions may be administered to patients in whom the affinity of hemoglobin for oxygen is abnormally high. For example, certain hemoglobinopathies, certain respiratory distress syndromes, e.g., respiratory distress syndromes in new born infants aggravated by high fetal hemoglobin levels, and conditions in which the availability of hemoglobin/ oxygen to the tissues is decreased (e.g., in ischemic conditions such as peripheral vascular disease, coronary occlusion, cerebral vascular accidents, or tissue transplant). The compounds and compositions may also be used to inhibit platelet aggregation, antithrombotic purposes, and wound healing. Topical application could be used for wound healing.

Additionally, the compounds and compositions of the present invention can be added to

whole blood or packed cells preferably at the time of storage or at the time of transfusion in order to facilitate the dissociation of oxygen from hemoglobin and improve the oxygen delivering capability of the blood. When blood is stored, the hemoglobin in the blood tends to increase its affinity for oxygen by losing 2,3-diphosphoglycerides. As described above, the compounds and compositions of this invention are capable of reversing and/or preventing the functional abnormality of hemoglobin observed when whole blood or packed cells are stored. The compounds and compositions may be added to whole blood or red blood cell fractions in a closed system using an appropriate reservoir in which the compound or composition is placed prior to storage or which is present in the anticoagulating solution in the blood collecting bag.

Administration to a patient can be achieved by intravenous or intraperitoneal injection where the dose and the dosing regiment is varied according to individual's sensitivity and the type of disease state being treated.

Solid tumors are oxygen deficient masses. The compounds, compositions and methods of this invention may be exploited to cause more oxygen to be delivered to tumors, increasing radical formation and thereby increasing tumor killing during radiation. In this context, such IHP-treated blood will only be used in conjunction with radiotherapy.

The compounds, compositions and methods of this invention may be exploited to cause more oxygen to be delivered at low blood flow and low temperatures, providing the ability to decrease or prevent the cellular damage, e.g., myocardial or neuronal, typically associated with these conditions.

The compounds, compositions and methods of this invention may be exploited to decrease the number of red blood cells required for treating hemorrhagic shock by increasing the efficiency with which they deliver oxygen.

Damaged tissues heal faster when there is better blood flow and increased oxygen tension. Therefore, the compounds, compositions and methods of this invention may be exploited to speed wound healing. Furthermore, by increasing oxygen delivery to wounded tissue, the compounds, compositions and methods of this invention may play a role in the destruction of infection causing bacteria at a wound.

The compounds, compositions and methods of this invention will be effective in enhancing the delivery oxygen to the brain, especially before complete occlusion and reperfusion injuries occur due to free radical formation. Furthermore, the compounds, compositions and methods of this invention of this invention should reduce the expansion of arterioles under both hypoxic and hypotensive conditions.

The compounds, compositions and methods of this invention of this invention should be

capable of increasing oxygen delivery to blocked arteries and surrounding muscles and tissues, thereby relieving the distress of angina attacks.

Acute respiratory disease syndrome (ARDS) is characterized by interstitial and/or alveolar edema and hemorrhage as well as perivascular lung edema associated with the hyaline membrane, proliferation of collagen fibers, and swollen epithelium with increased pinocytosis. The enhanced oxygen delivering capacity provided to RBCs by the compounds, compositions and methods of this invention can be used in the treatment and prevention of ARDS by militating against lower than normal oxygen delivery to the lungs.

There are several aspects of cardiac bypass surgery that make attractive the use of compounds or compositions or methods of the present invention. First, the compounds and compositions of the present invention act as neuroprotective agents. After cardiac bypass surgery, up to 50-70% of patients show some signs of cerebral ischemia based on tests of cognitive function. Up to 5% of these patients have evidence of stroke. Second, cardioplegia is the process of stopping the heart and protecting the heart from ischemia during heart surgery. Cardioplegia is performed by perfusing the coronary vessels with solutions of potassium chloride and bathing the heart in ice water. However, blood cardioplegia is also used. This is where potassium chloride is dissolved in blood instead of salt water. During surgery the heart is deprived of oxygen and the cold temperature helps slow down metabolism. Periodically during this process, the heart is perfused with the cardioplegia solution to wash out metabolites and reactive species. Cooling the blood increases the oxygen affinity of its hemoglobin, thus making oxygen unloading less efficient. However, treatment of blood cardioplegia with compounds or compositions of the present invention will counteract the effects of cold on oxygen affinity and make oxygen release to the ischemic myocardium more efficient, possibly improving cardiac function after the heart begins to beat again. Third, during bypass surgery the patient's blood is diluted for the process of pump prime. This hemodilution is essentially acute anemia. Because the compounds and compositions of the present invention make oxygen transport more efficient, their use during hemodilution (whether in bypass surgery or other surgeries, such as orthopedic or vascular) would enhance oxygenation of the tissues in an otherwise compromised condition. Finally, patients undergoing bypass surgery require blood transfusion after surgery. The use of compounds or compositions or methods of the present invention to make oxygen transport more efficient could obviate the need for transfusion, thus decreasing the cost of surgery and allowing the patient to avoid the risks associated with blood transfusion.

Definitions

For convenience, certain terms employed in the specification, examples, and appended

claims are collected here. As used throughout this specification and the claims, the following terms have the following meanings:

The term " hemoglobin" includes all naturally- and non-naturally-occurring hemoglobin.

The term " hemoglobin preparation" includes hemoglobin in a physiologically compatible carrier or lyophilized hemoglobin reconstituted with a physiologically compatible carrier, but does not include whole blood, red blood cells or packed red blood cells.

"Non-naturally-occurring hemoglobin" includes synthetic hemoglobin having an amino-acid sequence different from the amino-acid sequence of hemoglobin naturally existing within a cell, and chemically-modified hemoglobin. Such non-naturally-occurring mutant hemoglobin is not limited by its method of preparation, but is typically produced using one or more of several techniques known in the art, including, for example, recombinant DNA technology, transgenic DNA technology, protein synthesis, and other mutation-inducing methods.

"Chemically-modified hemoglobin" is a natural or non-natural hemoglobin molecule which is bonded to another chemical moiety. For example, a hemoglobin molecule can be bonded to pyridoxal-5'-phosphate, or other oxygen-affinity-modifying moiety to change the oxygen-binding characteristics of the hemoglobin molecule, to crosslinking agents to form crosslinked or polymerized hemoglobin, or to conjugating agents to form conjugated hemoglobin.

"Oxygen affinity" means the strength of binding of oxygen to a hemoglobin molecule. High oxygen affinity means hemoglobin does not readily release its bound oxygen molecules. The P50 is a measure of oxygen affinity.

"Cooperativity" refers to the sigmoidal oxygen-binding curve of hemoglobin, i.e., the binding of the first oxygen to one subunit within the tetrameric hemoglobin molecule enhances the binding of oxygen molecules to other unligated subunits. It is conveniently measured by the Hill coefficient (n[max]). For Hb A, n[max] = 3.0.

The term "treatment" is intended to encompass also prophylaxis, therapy and cure.

"Ischemia" means a temporary or prolonged lack or reduction of oxygen supply to an organ or skeletal tissue. Ischemia can be induced when an organ is transplanted, or by conditions such as septic shock and sickle cell anemia.

"Skeletal tissue" means the substance of an organic body of a skeletal organism consisting of cells and intercellular material, including but not limited to epithelium, the connective tissues (including blood, bone and cartilage), muscle tissue, and nerve tissue.

"Ischemic insult" means damage to an organ or skeletal tissue caused by ischemia.

"Subject" means any living organism, including humans, and mammals.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticulare, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

As used herein, the term "surgery" refers to the treatment of diseases, injuries, and deformities by manual or operative methods. Common surgical procedures include, but are not limited to, abdominal, aural, bench, cardiac, cineplastic, conservative, cosmetic, cytoreductive, dental, dentofacial, general, major, minor, Moh's, open heart, organ transplantation, orthopedic, plastic, psychiatric, radical, reconstructive, sonic, stereotactic, structural, thoracic, and veterinary surgery. The method of the present invention is suitable for patients that are to undergo any type of surgery dealing with any portion of the body, including but not limited to those described above, as well as any type of any general, major, minor, or minimal invasive surgery.

"Minimally invasive surgery" involves puncture or incision of the skin, or insertion of an instrument or foreign material into the body. Non-limiting examples of minimal invasive surgery include arterial or venous catheterization, transurethral resection, endoscopy (e.g., laparoscopy, bronchoscopy, uroscopy, pharyngoscopy, cystoscopy, hysteroscopy, gastroscopy, coloscopy, colposcopy, celioscopy, sigmoidoscopy, and orthoscopy), and angioplasty (e.g., balloon angioplasty, laser angioplasty, and percutaneous transluminal angioplasty).

The term "ED_{50}" means the dose of a drug which produces 50% of its maximum response or effect. Alternatively, the dose which produces a pre-determined response in 50% of test subjects or preparations.

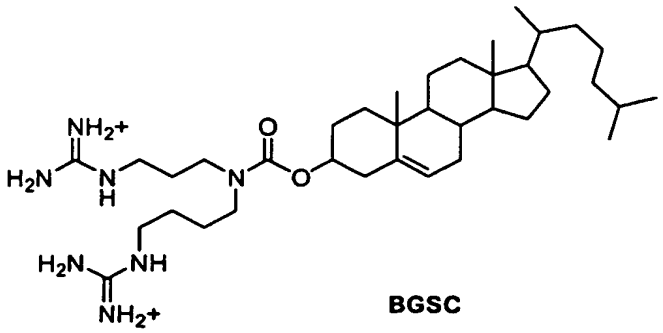
The term "LD_{50}" means the dose of a drug which is lethal in 50% of test subjects.

The term "therapeutic index" refers to the therapeutic index of a drug defined as LD_{50}/ED_{50}.

The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

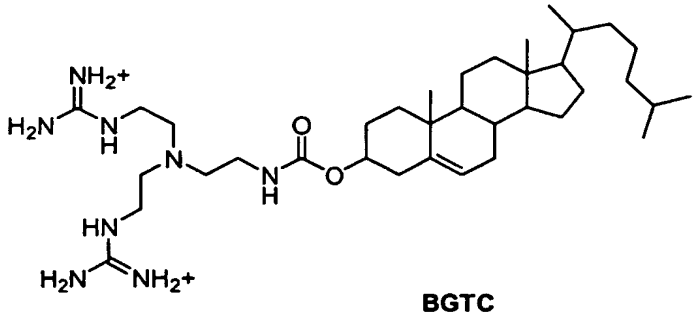
The term "structure-activity relationship (SAR)" refers to the way in which altering the molecular structure of drugs alters their interaction with a receptor, enzyme, etc.

The terms "bis-guanidinium-spermidine-cholesterol" and "BGSC" refer to the structure below.



BGSC

The terms "bis-guanidinium-tren-cholesterol" and "BGTC" refer to the structure below.



BGTC

The term "heteroatom" as used herein means an atom of any element other than carbon or hydrogen. Preferred heteroatoms are boron, nitrogen, oxygen, phosphorus, sulfur and selenium.

The term "electron-withdrawing group" is recognized in the art, and denotes the tendency of a substituent to attract valence electrons from neighboring atoms, i.e., the substituent is electronegative with respect to neighboring atoms. A quantification of the level of electron-withdrawing capability is given by the Hammett sigma (σ) constant. This well known constant is described in many references, for instance, J. March, Advanced Organic Chemistry, McGraw Hill Book Company, New York, (1977 edition) pp. 251-259. The Hammett constant values are generally negative for electron donating groups ($\sigma[P] = -0.66$ for NH_2) and positive for electron withdrawing groups ($\sigma[P] = 0.78$ for a nitro group), $\sigma[P]$ indicating para substitution. Exemplary electron-withdrawing groups include nitro, acyl, formyl, sulfonyl, trifluoromethyl, cyano, chloride, and the like. Exemplary electron-donating groups include amino, methoxy, and the like.

The term "alkyl" refers to the radical of saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl substituted

cycloalkyl groups, and cycloalkyl substituted alkyl groups. In preferred embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (e.g., C_1-C_{30} for straight chain, C_3-C_{30} for branched chain), and more preferably 20 or fewer. Likewise, preferred cycloalkyls have from 3-10 carbon atoms in their ring structure, and more preferably have 5, 6 or 7 carbons in the ring structure.

Moreover, the term "alkyl" (or "lower alkyl") as used throughout the specification, examples, and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, a halogen, a hydroxyl, a carbonyl (such as a carboxyl, an alkoxycarbonyl, a formyl, or an acyl), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an alkoxyl, a phosphoryl, a phosphonate, a phosphinate, an amino, an amido, an amidine, an imine, a cyano, a nitro, an azido, a sulfhydryl, an alkylthio, a sulfate, a sulfonate, a sulfamoyl, a sulfonamido, a sulfonyl, a heterocyclyl, an aralkyl, or an aromatic or heteroaromatic moiety. It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include substituted and unsubstituted forms of amino, azido, imino, amido, phosphoryl (including phosphonate and phosphinate), sulfonyl (including sulfate, sulfonamido, sulfamoyl and sulfonate), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), $-CF_3$, -CN and the like. Exemplary substituted alkyls are described below. Cycloalkyls can be further substituted with alkyls, alkenyls, alkoxys, alkylthios, aminoalkyls, carbonyl-substituted alkyls, $-CF_3$, -CN, and the like.

The term "aralkyl", as used herein, refers to an alkyl group substituted with an aryl group (e.g., an aromatic or heteroaromatic group).

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively.

Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to ten carbons, more preferably from one to six carbon atoms in its backbone structure. Likewise, "lower alkenyl" and "lower alkynyl" have similar chain lengths. Preferred alkyl groups are lower alkyls. In preferred embodiments, a substituent designated herein as alkyl is a lower alkyl.

The term "aryl" as used herein includes 5-, 6- and 7-membered single-ring aromatic groups that may include from zero to four heteroatoms, for example, benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles" or "heteroaromatics." The aromatic ring can be substituted at one or more ring positions with such substituents as described above, for example, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxyl, amino, nitro, sulfhydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, ester, heterocyclyl, aromatic or heteroaromatic moieties, $-CF_3$, -CN, or the like. The term "aryl" also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings (the rings are "fused rings") wherein at least one of the rings is aromatic, e.g., the other cyclic rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls.

The terms *ortho*, *meta* and *para* apply to 1,2-, 1,3- and 1,4-disubstituted benzenes, respectively. For example, the names 1,2-dimethylbenzene and *ortho*-dimethylbenzene are synonymous.

The terms "heterocyclyl" or "heterocyclic group" refer to 3- to 10-membered ring structures, more preferably 3- to 7-membered rings, whose ring structures include one to four heteroatoms. Heterocycles can also be polycycles. Heterocyclyl groups include, for example, thiophene, thianthrene, furan, pyran, isobenzofuran, chromene, xanthene, phenoxathiin, pyrrole, imidazole, pyrazole, isothiazole, isoxazole, pyridine, pyrazine, pyrimidine, pyridazine, indolizine, isoindole, indole, indazole, purine, quinolizine, isoquinoline, quinoline, phthalazine, naphthyridine, quinoxaline, quinazoline, cinnoline, pteridine, carbazole, carboline, phenanthridine, acridine, pyrimidine, phenanthroline, phenazine, phenarsazine, phenothiazine, furazan, phenoxazine, pyrrolidine, oxolane, thiolane, oxazole, piperidine, piperazine, morpholine, lactones, lactams such as azetidinones and pyrrolidinones, sultams, sultones, and the like. The heterocyclic ring can be substituted at one or more positions with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, $-CF_3$, -CN, or the like.

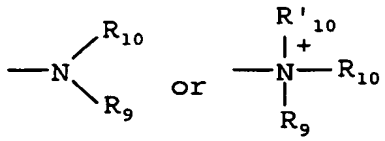
The terms "polycyclyl" or "polycyclic group" refer to two or more rings (e.g., cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocyclyls) in which two or more

carbons are common to two adjoining rings, e.g., the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycycle can be substituted with such substituents as described above, as for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, $-CF_3$, -CN, or the like.

The term "carbocycle", as used herein, refers to an aromatic or non-aromatic ring in which each atom of the ring is carbon.

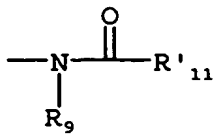
As used herein, the term "nitro" means $-NO_2$; the term "halogen" designates -F, -Cl, -Br or -I; the term "sulfhydryl" means -SH; the term "hydroxyl" means -OH; and the term "sulfonyl" means $-SO_2-$.

The terms "amine" and "amino" are art-recognized and refer to both unsubstituted and substituted amines, e.g., a moiety that can be represented by the general formula:



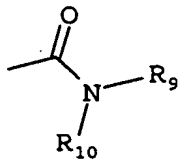
wherein R_9, R_{10} and R'_{10} each independently represent a hydrogen, an alkyl, an alkenyl, $-(CH_2)_m-R_8$, or R_9 and R_{10} taken together with the N atom to which they are attached complete a heterocycle having from 4 to 8 atoms in the ring structure; R_8 represents an aryl, a cycloalkyl, a cycloalkenyl, a heterocycle or a polycycle; and m is zero or an integer in the range of 1 to 8. In preferred embodiments, only one of R_9 or R_{10} can be a carbonyl, e.g., R_9, R_{10} and the nitrogen together do not form an imide. In even more preferred embodiments, R_9 and R_{10} (and optionally R'_{10}) each independently represent a hydrogen, an alkyl, an alkenyl, or $-(CH_2)_m-R_8$. Thus, the term "alkylamine" as used herein means an amine group, as defined above, having a substituted or unsubstituted alkyl attached thereto, i.e., at least one of R_9 and R_{10} is an alkyl group.

The term "acylamino" is art-recognized and refers to a moiety that can be represented by the general formula:



wherein R_9 is as defined above, and R'_{11} represents a hydrogen, an alkyl, an alkenyl or $-(CH_2)_m-R_8$, where m and R_8 are as defined above.

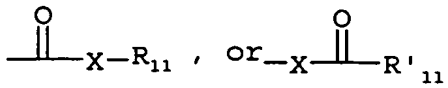
The term "amido" is art recognized as an amino-substituted carbonyl and includes a moiety that can be represented by the general formula:



wherein R_9, R_{10} are as defined above. Preferred embodiments of the amide will not include imides which may be unstable.

The term "alkylthio" refers to an alkyl group, as defined above, having a sulfur radical attached thereto. In preferred embodiments, the "alkylthio" moiety is represented by one of -S-alkyl, -S-alkenyl, -S-alkynyl, and $-S-(CH_2)_m-R_8$, wherein m and R_8 are defined above. Representative alkylthio groups include methylthio, ethyl thio, and the like.

The term "carbonyl" is art recognized and includes such moieties as can be represented by the general formula:

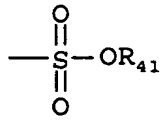


wherein X is a bond or represents an oxygen or a sulfur, and R_{11} represents a hydrogen, an alkyl, an alkenyl, $-(CH_2)_m-R_8$ or a pharmaceutically acceptable salt, R'_{11} represents a hydrogen, an alkyl, an alkenyl or $-(CH_2)_m-R_8$, where m and R_8 are as defined above. Where X is an oxygen and R_{11} or R'_{11} is not hydrogen, the formula represents an "ester". Where X is an oxygen, and R_{11} is as defined above, the moiety is referred to herein as a carboxyl group, and particularly when R_{11} is a hydrogen, the formula represents a "carboxylic acid". Where X is an oxygen, and R'_{11} is hydrogen, the formula represents a "formate". In general, where the oxygen atom of the above formula is replaced by sulfur, the formula represents a "thiolcarbonyl" group. Where X is a sulfur and R_{11} or R'_{11} is not hydrogen, the formula represents a "thiolester." Where X is a sulfur and R_{11} is hydrogen, the formula represents a "thiolcarboxylic acid." Where X is a sulfur and R_{11}' is hydrogen, the formula represents a "thiolformate." On the other hand, where X is a

bond, and R_{11} is not hydrogen, the above formula represents a "ketone" group. Where X is a bond, and R_{11} is hydrogen, the above formula represents an "aldehyde" group.

The terms "alkoxyl" or "alkoxy" as used herein refers to an alkyl group, as defined above, having an oxygen radical attached thereto. Representative alkoxyl groups include methoxy, ethoxy, propyloxy, tert-butoxy and the like. An "ether" is two hydrocarbons covalently linked by an oxygen. Accordingly, the substituent of an alkyl that renders that alkyl an ether is or resembles an alkoxyl, such as can be represented by one of -O-alkyl, -O-alkenyl, -O-alkynyl, -O-$(CH_2)_m$-R_8, where m and R_8 are described above.

The term "sulfonate" is art recognized and includes a moiety that can be represented by the general formula:

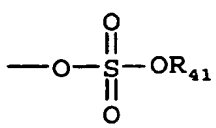


in which R_{41} is an electron pair, hydrogen, alkyl, cycloalkyl, or aryl.

The terms triflyl, tosyl, mesyl, and nonaflyl are art-recognized and refer to trifluoromethanesulfonyl, *p*-toluenesulfonyl, methanesulfonyl, and nonafluorobutanesulfonyl groups, respectively. The terms triflate, tosylate, mesylate, and nonaflate are art-recognized and refer to trifluoromethanesulfonate ester, *p*-toluenesulfonate ester, methanesulfonate ester, and nonafluorobutanesulfonate ester functional groups and molecules that contain said groups, respectively.

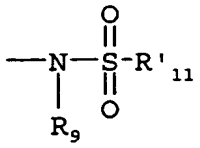
The abbreviations Me, Et, Ph, Tf, Nf, Ts, and Ms represent methyl, ethyl, phenyl, trifluoromethanesulfonyl, nonafluorobutanesulfonyl, *p*-toluenesulfonyl and methanesulfonyl, respectively. A more comprehensive list of the abbreviations utilized by organic chemists of ordinary skill in the art appears in the first issue of each volume of the *Journal of Organic Chemistry*; this list is typically presented in a table entitled Standard List of Abbreviations. The abbreviations contained in said list, and all abbreviations utilized by organic chemists of ordinary skill in the art are hereby incorporated by reference.

The term "sulfate" is art recognized and includes a moiety that can be represented by the general formula:



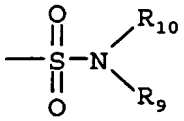
in which R_{41} is as defined above.

The term "sulfonamido" is art recognized and includes a moiety that can be represented by the general formula:



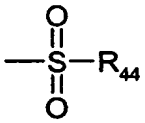
in which R_9 and R'_{11} are as defined above.

The term "sulfamoyl" is art-recognized and includes a moiety that can be represented by the general formula:



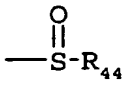
in which R_9 and R_{10} are as defined above.

The term "sulfonyl", as used herein, refers to a moiety that can be represented by the general formula:



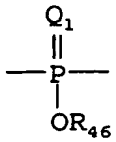
in which R_{44} is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, or heteroaryl.

The term "sulfoxido" as used herein, refers to a moiety that can be represented by the general formula:

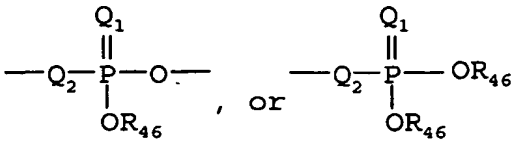


in which R_{44} is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aralkyl, or aryl.

A "phosphoryl" can in general be represented by the formula:



wherein Q_1 represented S or O, and R_{46} represents hydrogen, a lower alkyl or an aryl. When used to substitute, e.g., an alkyl, the phosphoryl group of the phosphorylalkyl can be represented by the general formula:



wherein Q_1 represented S or O, and each R_{46} independently represents hydrogen, a lower alkyl or an aryl, Q_2 represents O, S or N. When Q_1 is an S, the phosphoryl moiety is a "phosphorothioate".

Analogous substitutions can be made to alkenyl and alkynyl groups to produce, for example, aminoalkenyls, aminoalkynyls, amidoalkenyls, amidoalkynyls, iminoalkenyls, iminoalkynyls, thioalkenyls, thioalkynyls, carbonyl-substituted alkenyls or alkynyls.

As used herein, the definition of each expression, e.g. alkyl, m, n, etc., when it occurs more than once in any structure, is intended to be independent of its definition elsewhere in the same structure.

It will be understood that "substitution" or "substituted with" includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, e.g., which does not spontaneously undergo transformation such as by rearrangement, cyclization, elimination, etc.

As used herein, the term "substituted" is contemplated to include all permissible substituents of organic compounds. In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds. Illustrative substituents include, for example, those described herein above. The permissible substituents can be one or more and the same or different for appropriate organic compounds. For purposes of this invention, the heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valences of the heteroatoms. This invention is not intended to be limited in any manner by the permissible substituents of organic compounds.

The phrase "protecting group" as used herein means temporary substituents which protect a potentially reactive functional group from undesired chemical transformations. Examples of such protecting groups include esters of carboxylic acids, silyl ethers of alcohols, and acetals and ketals of aldehydes and ketones, respectively. The field of protecting group chemistry has been reviewed (Greene, T.W.; Wuts, P.G.M. *Protective Groups in Organic Synthesis*, 2nd ed.; Wiley: New York, 1991).

Certain compounds of the present invention may exist in particular geometric or stereoisomeric forms. The present invention contemplates all such compounds, including *cis*- and *trans*-isomers, *R*- and *S*-enantiomers, diastereomers, (D)-isomers, (L)-isomers, the racemic mixtures thereof, and other mixtures thereof, as falling within the scope of the invention. Additional asymmetric carbon atoms may be present in a substituent such as an alkyl group. All such isomers, as well as mixtures thereof, are intended to be included in this invention.

If, for instance, a particular enantiomer of a compound of the present invention is desired, it may be prepared by asymmetric synthesis, or by derivation with a chiral auxiliary, where the resulting diastereomeric mixture is separated and the auxiliary group cleaved to provide the pure desired enantiomers. Alternatively, where the molecule contains a basic functional group, such as amino, or an acidic functional group, such as carboxyl, diastereomeric salts are formed with an appropriate optically-active acid or base, followed by resolution of the diastereomers thus formed by fractional crystallization or chromatographic means well known in the art, and subsequent recovery of the pure enantiomers.

Contemplated equivalents of the compounds described above include compounds which otherwise correspond thereto, and which have the same general properties thereof, wherein one or more simple variations of substituents are made which do not adversely affect the efficacy of the compound. In general, the compounds of the present invention may be prepared by the methods illustrated in the general reaction schemes as, for example, described below, or by modifications thereof, using readily available starting materials, reagents and conventional synthesis procedures. In these reactions, it is also possible to make use of variants which are in themselves known, but are not mentioned here.

For purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 67th Ed., 1986-87, inside cover. Also for purposes of this invention, the term "hydrocarbon" is contemplated to include all permissible compounds having at least one hydrogen and one carbon atom. In a broad aspect, the permissible hydrocarbons include acyclic and cyclic, branched and

unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic organic compounds which can be substituted or unsubstituted.

Compounds of the Invention.

In 1996, Vigneron et al. (*Proc. Natl. Acad. Sci. USA* **1996**, *93*, 9682-86) disclosed the synthesis of two cationic lipids, bis-guanidinium-spermidine-cholesterol (BGSC) and bis-guanidinium-tren-cholesterol (BGTC), and their testing as artificial vectors for gene transfer. Furthermore, Lehn et al. have filed a PCT patent application (WO 97/31935) directed toward novel amidinium-bearing cholesterol derivatives and pharmaceutical compositions thereof, which are particularly useful in gene therapy for transferring therapeutic genes into cells. These compounds combine the membrane compatible features of cholesterol with the favorable structural and pK_a features of the guanidinium functions for binding phosphate and carboxylate groups. A micellar solution of BGTC was reported to efficiently transfect DNA into a variety of mammalian cell lines. In addition, both BGTC and BGSC displayed high transfection activity when formulated as liposomes with the neutral phospholipid, dioleoylphosphatidyl ethanolamine. These results revealed the usefulness for gene transfer of cholesterol derivatives bearing guanidinium groups. Additionally, published PCT applications, WO 96/01840, "Cationic Lipids for Delivery of Nucleic Acids to Cells", and WO 96/18372, "Cationic Amphiphiles and Plasmids for Intracellular Delivery of Therapeutic Molecules", describe compounds, compositions and methods for the delivery of anions into the cytoplasm of mammalian cells, based on the use of complexes consisting essentially of cationic lipophilic water-soluble molecules and the anion to be delivered into the cytoplasm.

The guanidinium moiety has been shown to be well-suited to complexation of carboxylates and phosphates, due to its high pKa value (relative to that of the ammonium moiety), and its ability to form two well-organized, strong zwitterionic hydrogen bonds with those functional groups. See Echavarren et al. *Helv. Chim. Acta* **1988**, *71*, 685-93; Echavarren et al. *J. Am. Chem. Soc.* **1989**, *111*, 4994-95; and Dietrich et al. *Helv. Chim. Acta* **1979**, *62*, 2763-87). Furthermore, Dietrich et al. (*J. Chem. Soc., Chem. Commun.* **1978**, 934) have disclosed a general method for the introduction of guanidinium groups into macrocyclic molecules.

Synthetic "vectors", e.g., BGTC and BGSC, represent an attractive means for delivery of small anionic molecules into the cytoplasm of mammalian cells. Various cationic lipids have been shown to induce efficient transfection of a large variety of eukaryotic cells. Most of them have been formulated as liposomes containing two lipid species, a cationic amphiphile and a neutral phospholipid, typically dioleoylphosphatidyl ethanolamine (DOPE). However, some lipids (e.g., lipopolyamines) have been used directly as cationic amphiphilic reagents in solution.

The spontaneous formation of DNA/lipid aggregates *in vitro* is in any case due to ionic interactions between the positively charged cationic lipid and the negatively charged phosphate groups of the DNA; residual positive charges on the aggregates presumably mediate their binding to negatively charged (sialic acid) residues on cell surfaces.

Several years ago, it was discovered that the antilipidemic drug clofibric acid lowered the oxygen affinity of hemoglobin solutions (Abraham et al., J. Med. Chem. 25, 1015 (1982), and Abraham et al., Proc. Natl. Acad. Sci. USA 80, 324 (1983)). Bezafibrate, another antilipidemic drug, was later found to be much more effective in lowering the oxygen affinity of hemoglobin solutions and suspensions of fresh, intact red cells (Perutz et al., Lancet, 881, Oct. 15, 1983). Subsequently, X-ray crystallographic studies have demonstrated that clofibric acid and bezafibrate bind to the same sites in the central water cavity of deoxyhemoglobin, and that one bezafibrate molecule will span the sites occupied by two clofibric acid molecules. Bezafibrate and clofibric acid act by stabilizing the deoxy structure of hemoglobin, shifting the allosteric equilibrium toward the low affinity deoxy form. Bezafibrate and clofibric acid do not bind in any specific manner to either oxy- or carbonmonoxyhemoglobin.

In later investigations, a series of urea derivatives [2-[4-[[(arylamino)carbonyl]-amino]phenoxy]-2-methylpropionic acids] was discovered that has greater allosteric potency than bezafibrate at stabilizing the deoxy structure of hemoglobin and shifting the allosteric equilibrium toward the low oxygen affinity form (Lalezari, Proc. Natl. Acad. Sci. USA 85, 6117 (1988)).

It has been determined that certain allosteric hemoglobin modifier compounds are hydrophobic molecules that can be bound to the body's neutral fat deposits and lipophilic receptors sites, thus lowering their potency due to a decreased concentration in RBCs. Administration of a hydrophobic compound, such as a mixture of anesthetic molecules, will saturate the body's neutral fat deposits and lipophilic receptor sites, and thereby increase the concentration of this type of allosteric modifiers in RBCs, where higher concentrations of effector will increase its ability to interact with hemoglobin, causing delivery of more oxygen.

Ligands for the allosteric site of hemoglobin include 2,3-diphosphoglycerate (DPG), inositol hexakisphosphate (IHP), bezafibrate (Bzf), LR16 and L35 (two recently synthesized derivatives of Bzf), and pyridoxal phosphate. Additionally, hemoglobin's affinity for oxygen can be modulated through electrostatic interactions with chloride and/or organophosphate anions present in RBCs. These effectors, which bind preferentially to the deoxy-Hb tetramers at a distance from the heme groups, play a major role in the adaptation of the respiratory properties of hemoglobin to either allometric-dependent oxygen needs or to various hypoxic environments.

Additionally, protons and carbon dioxide are physiological regulators for the oxygen affinity of hemoglobin. The heterotropic allosteric interaction between the non-heme ligands and oxygen, collectively called the Bohr effect, facilitates not only the transport of oxygen but also the exchange of carbon dioxide.

The present invention relates to compositions, and methods of use thereof, consisting essentially of a cationic, lipophilic, water-soluble molecule (e.g., a bis-guanidinium cholesterol), and an anionic ligand for the allosteric site of hemoglobin, e.g., inositol hexaphosphate (IHP). In certain embodiments, the present invention is related to compounds and compositions thereof which deliver into erythrocytes allosteric modifiers of hemoglobin *in vivo*. Additionally, the invention is directed to the use of the compounds or compositions thereof that are effective in delivering into erythrocytes allosteric modifiers of hemoglobin, lowering the oxygen affinity state in red blood cell suspensions and whole blood. It is an object of this invention to provides methods for delivering into erythrocytes allosteric modifiers of hemoglobin in whole blood and *in vivo*, utilizing compounds or compositions thereof that do not lose their effectiveness in the presence of normal concentrations of the remaining components of whole blood.

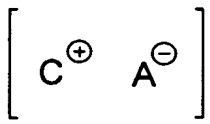
Additionally, the present invention is directed toward the design of water-soluble membrane compatible molecules bearing novel cationic moieties, e.g., guanidinium-bearing sterol derivatives. These molecules form complexes with anionic molecules, e.g., ligands for the allosteric site of hemoglobin; such complexes are useful for the deliver of said anionic molecules into the cytoplasm of mammalian cells, e.g., erythrocytes.

The compounds and compositions thereof of the present invention may be used to prepare cationic liposomes, and should also be effective directly as aqueous (micellar) solutions, thereby avoiding the need to prepare cationic liposomes and the difficulties associated with them. The cationic membrane compatible molecules are designed to form complexes with anionic molecules, e.g., ligands for the allosteric site of hemoglobin. These complexes will react with mammalian cells in vitro and/or in vivo to deliver their anionic component into the cytoplasm of the cells.

The guanidinium group of the cationic component of the compounds of the present invention is particularly well suited for interaction with the phosphate residues of IHP and congeners thereof because a pair of hydrogen bonds can be established between the two moieties. We report here the use of bisguanidinium cholesterol lipids for the efficient delivery into mammalian erythrocytes of phosphate-containing ligands for the allosteric site of hemoglobin. Our data demonstrate the usefulness, convenience, and versatility of cationic cholesterol derivatives with guanidinium polar head groups for delivery of small anionic molecules into the

cytoplasm of mammalian cells.

In certain embodiments, the compounds of the present invention are represented by generalized structure 1:



1

wherein

C+ represents a lipophilic water-soluble molecule bearing at least one positive charge; and

A- represents a ligand for a mammalian cellular receptor, wherein said ligand bears at least one negative charge.

In certain embodiments, the compounds of the present invention are represented by generalized structure 1, and the attendant definitions, wherein A- is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention are represented by generalized structure 1, and the attendant definitions, wherein C+ comprises at least one cationic functional group selected from the group consisting of guanidinium, imidazolium, 1,2-diammoniumethylene, 1,8-diammoniumnaphthyl, and 2,2'-bipyridinium.

In certain embodiments, the compounds of the present invention are represented by generalized structure 1, and the attendant definitions, wherein C+ comprises at least one guanidinium moiety.

In certain embodiments, the compounds of the present invention are represented by generalized structure 1, and the attendant definitions, wherein C+ comprises two guanidinium moieties.

In certain embodiments, the compounds of the present invention are represented by generalized structure 1, and the attendant definitions, wherein C+ comprises at least one cationic functional group selected from the group consisting of guanidinium, imidazolium, 1,2-

diammoniumethylene, 1,8-diammoniumnaphthyl, and 2,2'-bipyridinium; and A- is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention are represented by generalized structure 1, and the attendant definitions, wherein C+ comprises at least one guanidinium moiety; and A- is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention are represented by generalized structure 1, and the attendant definitions, wherein C+ comprises two guanidinium moieties; and A- is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of a lipophilic water-soluble molecule, wherein said lipophilic water-soluble molecule comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid, phosphoric acid, phosphonic acid, sulfuric acid, or sulfonic acid moiety.

In certain embodiments, the compounds of the present invention consist essentially of a lipophilic water-soluble molecule, wherein said lipophilic water-soluble molecule comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid or phosphoric acid moiety.

In certain embodiments, the compounds of the present invention consist essentially of a lipophilic water-soluble molecule, wherein said lipophilic water-soluble molecule comprises at least one guanidine or guanidinium moiety; and a phosphorylated inositol.

In certain embodiments, the compounds of the present invention consist essentially of a lipophilic water-soluble molecule, wherein said lipophilic water-soluble molecule comprises at least one guanidine or guanidinium moiety; and IHP.

In certain embodiments, the compounds of the present invention consist essentially of a lipophilic water-soluble molecule, wherein said lipophilic water-soluble molecule comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid, phosphoric acid, phosphonic acid, sulfuric acid, or sulfonic acid moiety, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of a lipophilic water-soluble molecule, wherein said lipophilic water-soluble molecule comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid or phosphoric acid moiety, wherein said second molecule is a ligand for the

allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of a lipophilic water-soluble molecule, wherein said lipophilic water-soluble molecule comprises at least one guanidine or guanidinium moiety; and a phosphorylated inositol, wherein said phosphorylated inositol is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of a sterol, wherein said sterol comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid, phosphoric acid, phosphonic acid, sulfuric acid, or sulfonic acid moiety.

In certain embodiments, the compounds of the present invention consist essentially of a sterol, wherein said sterol comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid or phosphoric acid moiety.

In certain embodiments, the compounds of the present invention consist essentially of a sterol, wherein said sterol comprises at least one guanidine or guanidinium moiety; and a phosphorylated inositol.

In certain embodiments, the compounds of the present invention consist essentially of a sterol, wherein said sterol comprises at least one guanidine or guanidinium moiety; and IHP.

In certain embodiments, the compounds of the present invention consist essentially of a sterol, wherein said sterol comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid, phosphoric acid, phosphonic acid, sulfuric acid, or sulfonic acid moiety, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of a sterol, wherein said sterol comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid or phosphoric acid moiety, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of a sterol, wherein said sterol comprises at least one guanidine or guanidinium moiety; and a phosphorylated inositol, wherein said phosphorylated inositol is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of cholesterol, wherein said cholesterol comprises at least one guanidine or guanidinium moiety;

and a second molecule comprising at least one carboxylic acid, phosphoric acid, phosphonic acid, sulfuric acid, or sulfonic acid moiety.

In certain embodiments, the compounds of the present invention consist essentially of cholesterol, wherein said cholesterol comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid or phosphoric acid moiety.

In certain embodiments, the compounds of the present invention consist essentially of cholesterol, wherein said cholesterol comprises at least one guanidine or guanidinium moiety; and a phosphorylated inositol.

In certain embodiments, the compounds of the present invention consist essentially of cholesterol, wherein said cholesterol comprises at least one guanidine or guanidinium moiety; and IHP.

In certain embodiments, the compounds of the present invention consist essentially of cholesterol, wherein said cholesterol comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid, phosphoric acid, phosphonic acid, sulfuric acid, or sulfonic acid moiety, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of cholesterol, wherein said cholesterol comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid or phosphoric acid moiety, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of cholesterol, wherein said cholesterol comprises at least one guanidine or guanidinium moiety; and a phosphorylated inositol, wherein said phosphorylated inositol is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of BGSC or BGTC; and a second molecule comprising at least one carboxylic acid, phosphoric acid, phosphonic acid, sulfuric acid, or sulfonic acid moiety.

In certain embodiments, the compounds of the present invention consist essentially of BGSC or BGTC; and a second molecule comprising at least one carboxylic acid or phosphoric acid moiety.

In certain embodiments, the compounds of the present invention consist essentially of BGSC or BGTC; and a phosphorylated inositol.

In certain embodiments, the compounds of the present invention consist essentially of

BGSC or BGTC; and IHP.

In certain embodiments, the compounds of the present invention consist essentially of BGSC or BGTC; and a second molecule comprising at least one carboxylic acid, phosphoric acid, phosphonic acid, sulfuric acid, or sulfonic acid moiety, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of BGSC or BGTC; and a second molecule comprising at least one carboxylic acid or phosphoric acid moiety, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of BGSC or BGTC; and a phosphorylated inositol, wherein said phosphorylated inositol is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of BGTC; and a second molecule comprising at least one carboxylic acid, phosphoric acid, phosphonic acid, sulfuric acid, or sulfonic acid moiety.

In certain embodiments, the compounds of the present invention consist essentially of BGTC; and a second molecule comprising at least one carboxylic acid or phosphoric acid moiety.

In certain embodiments, the compounds of the present invention consist essentially of BGTC; and a phosphorylated inositol.

In certain embodiments, the compounds of the present invention consist essentially of BGTC; and IHP.

In certain embodiments, the compounds of the present invention consist essentially of BGTC; and a second molecule comprising at least one carboxylic acid, phosphoric acid, phosphonic acid, sulfuric acid, or sulfonic acid moiety, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of BGTC; and a second molecule comprising at least one carboxylic acid or phosphoric acid moiety, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

In certain embodiments, the compounds of the present invention consist essentially of BGTC; and a phosphorylated inositol, wherein said phosphorylated inositol is a ligand for the allosteric site of hemoglobin.

In certain embodiments, a compound of the present invention is a component of a

pharmaceutical composition.

In certain embodiments, a compound of the present invention is formulated in a liposome.

In certain embodiments, a compound of the present invention is formulated for intravenous administration.

In certain embodiments, the method of the present invention comprises the step of administering to a subject a compound or composition of the present invention.

In certain embodiments, the method of the present invention comprises the step of administering to a subject a compound or composition of the present invention, wherein said administration is intravenous.

In certain embodiments, the method of the present invention comprises the step of administering to a subject experiencing ischemia a compound or composition of the present invention.

In certain embodiments, the method of the present invention comprises the step of administering to a subject experiencing ischemia a compound or composition of the present invention, wherein said administration is intravenous.

In certain embodiments, the method of the present invention comprises the step of administering to a subject experiencing cardiac arrhythmia a compound or composition of the present invention.

In certain embodiments, the method of the present invention comprises the step of administering to a subject experiencing cardiac arrhythmia a compound or composition of the present invention, wherein said administration is intravenous.

In certain embodiments, the method of the present invention comprises the step of administering to a subject experiencing a heart attack a compound or composition of the present invention.

In certain embodiments, the method of the present invention comprises the step of administering to a subject experiencing a heart attack a compound or composition of the present invention, wherein said administration is intravenous.

In certain embodiments, the method of the present invention comprises the step of administering to a subject experiencing a stroke a compound or composition of the present invention.

In certain embodiments, the method of the present invention comprises the step of administering to a subject experiencing a stroke a compound or composition of the present invention, wherein said administration is intravenous.

In certain embodiments, the method of the present invention comprises the step of administering to a subject experiencing hypoxia a compound or composition of the present invention.

In certain embodiments, the method of the present invention comprises the step of administering to a subject experiencing hypoxia a compound or composition of the present invention, wherein said administration is intravenous.

In certain embodiments, the method of the present invention comprises the step of administering to a subject afflicted with sickle cell anemia a compound or composition of the present invention.

In certain embodiments, the method of the present invention comprises the step of administering to a subject afflicted with sickle cell anemia a compound or composition of the present invention, wherein said administration is intravenous.

In certain embodiments, the method of the present invention comprises the step of administering to a subject suffering from hypotension a compound or composition of the present invention.

In certain embodiments, the method of the present invention comprises the step of administering to a subject suffering from hypotension a compound or composition of the present invention, wherein said administration is intravenous.

In certain embodiments, the method of the present invention comprises the step of administering to a subject suffering from arteriosclerosis a compound or composition of the present invention.

In certain embodiments, the method of the present invention comprises the step of administering to a subject suffering from arteriosclerosis a compound or composition of the present invention, wherein said administration is intravenous.

In certain embodiments, the method of the present invention comprises the step of administering to a subject suffering from altitude sickness a compound or composition of the present invention.

In certain embodiments, the method of the present invention comprises the step of administering to a subject suffering from altitude sickness a compound or composition of the present invention, wherein said administration is intravenous.

In certain embodiments, the method of the present invention comprises the step of adding to mammalian blood a compound or composition of the present invention.

In certain embodiments, the method of the present invention comprises the step of adding to plasma comprising mammalian erythrocytes a compound or composition of the present

invention.

Pharmaceutical Compositions

In another aspect, the present invention provides pharmaceutically acceptable compositions which comprise a therapeutically-effective amount of one or more of the compounds described above, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; or (4) intravaginally or intrarectally, for example, as a pessary, cream or foam.

The phrase "therapeutically-effective amount" as used herein means that amount of a compound, material, or composition comprising a compound of the present invention which is effective for producing some desired therapeutic effect in at least a sub-population of cells in an animal at a reasonable benefit/risk ratio applicable to any medical treatment.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and

suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

As set out above, certain embodiments of the present compounds contain a cationic or basic functional group, such as an ammonium ion, or amino or alkylamino group, and are, thus, capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable acids, e.g., a ligand for the allosteric site of hemoglobin such as inositol hexaphosphate (IHP). These salts can be prepared *in situ* during the final isolation and purification of the compounds of the invention, or by separately reacting a first salt of the invention with a second suitable organic or inorganic salt, and isolating the new salt thus formed. For example, the chloride salt of a guanidinylated sterol may be combined with the potassium salt of IHP to give potassium chloride and a salt of the present invention comprising the guanidinylated sterol and IHP. In this regard, representative anions include bromide, chloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, napthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate; and representative cations include sodium, potassium, lithium, cesium, magnesium, calcium, and barium.

In certain cases, the compounds of the present invention may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable bases. The term "pharmaceutically-acceptable salts" in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of compounds of the present invention. These salts can likewise be prepared *in situ* during the final isolation and purification of the compounds, or by separately reacting the purified compound in its free acid form with a suitable base.

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium

metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations of the present invention include, but are not limited to, those suitable for parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. For example, the amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present invention as an active ingredient. A compound of the present invention may also be administered as a bolus, electuary or paste.

In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically-acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating

agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration of the compounds of the invention include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents

commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations of the pharmaceutical compositions of the invention for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of a compound of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to an active compound of this invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to a compound of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as

chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of a compound of the present invention to the body. Such dosage forms can be made by dissolving or dispersing the compound in the proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the compound in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more compounds of the invention in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms upon the subject compounds may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, or phenol sorbic acid. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption, such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug, e.g., from a subcutaneous or intramuscular injection. This goal may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution

which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the subject compounds in biodegradable polymers, such as polylactide-polyglycolide. In certain embodiments, the monomers of the biodegradable polymer comprise the functionalized sterol of the salts of the present invention, so that as the polymer biodegrades the salt of the present invention is released over a desired period of time. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

The preparations of the present invention may be given orally, parenterally, topically, or rectally. They are of course given in forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, etc. administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. Intravenous administrations are preferred.

These compounds may be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular compound of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In general, a suitable dose of a compound of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above.

If desired, the effective daily dose of the active compound may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition).

In another aspect, the present invention provides pharmaceutically acceptable compositions which comprise a therapeutically-effective amount of one or more of the subject compounds, as described above, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; or (4) intravaginally or intravectally, for example, as a pessary, cream or foam.

The compounds according to the invention may be formulated for administration in any convenient way for use in human or veterinary medicine, by analogy with other pharmaceuticals.

The patient receiving this treatment is any animal in need, including primates, in particular humans, and other mammals such as equines, cattle, swine and sheep; and poultry and pets in general.

The compound of the invention can be administered as such or in admixtures with pharmaceutically acceptable carriers and can also be administered in conjunction with antimicrobial agents such as penicillins, cephalosporins, aminoglycosides and glycopeptides. Conjunctive therapy, thus includes sequential, simultaneous and separate administration of the active compound in a way that the therapeutical effects of the first administered one is not entirely disappeared when the subsequent is administered.

Administration of the Compounds of the Present Invention

Many techniques currently exist for delivering drugs or other medicaments to body tissue. These include, among possible others, oral administration, injection directly into body tissue such as through an intramuscular injection or the like, topical or transcutaneous administration where the drug is passively absorbed, or caused to pass, into or across the skin or other surface tissue and intravenous administration which involves introducing a selected drug directly into the blood stream.

Except for topical or transcutaneous administration, the above drug delivery systems tend to be systemic. In other words, administration of the drug is delivered throughout the body by the blood stream.

Several devices have been developed for the purpose of accessing specific body lumens or passageways (i.e., blood vessels, gastrointestinal tract, urinary tract) and delivering therapeutic agents transmurally to specific subregions of tissue. A double-balloon catheter has been used to administer agents to the area confined by the balloons. A disadvantage of this system is that drugs may be lost through communicating vessels between the balloons. Alternatively, a perforated balloon has been developed to deliver agents directly into the vessel wall. A major disadvantage with both of these systems in certain desired applications is that the drug is delivered radially in all directions.

It is contemplated that other transport forces could also be used either with or in lieu of pressure to enhance or otherwise control the speed of drug transport. For example, one method could utilize DMSO as a carrier to transport a fixative or drug through the vessel wall. Other fluid diffusion enhancement compositions include propylene glycol, azone and ionic or non-ionic surfactants.

The parenteral administration of medical liquids is an established clinical practice. The liquids are administered particularly intravenously, and the practice is used extensively as an

integral part of the daily treatment of medical and surgical patients. The liquids commonly administered include blood and blood substitutes, dextrose solution, electrolyte solution, and saline. Generally, the liquids are administered from an intravenous delivery system having a container suspended above the patient, with the liquid flowing through a catheter hypodermic needle set to the patient.

The administration of liquids intravenously is a valuable and important component that contributes to the optimal care of the patient; however, it does not always provide a satisfactory means and method for administering concomitantly therewith a beneficial agent. Presently, a beneficial agent is often administered intravenously by (1) temporarily removing the intravenous system and halting the flow of liquid, and then intravenously administering the agent to the patient followed by reinserting the intravenous system into the patient; (2) the agent is added to the liquid in the container and then carried by the flow of the liquid to the patient; (3) agent is added to a liquid in a separate container called a partial fill that is connected to the primary intravenous line through which line the agent is carried by the flow of liquid to the patient; (4) an agent is contained in a piggyback vial into which is introduced an intravenous fluid, with the vital subsequently connected to the primary line through which the drug is administered to the patient; or (5) the agent is administered by a pump that exerts a force on a liquid containing agent for intravenously adminstering the liquid containing the agent. While these techniques are used, they have some disadvantages. For example, the administration of an agent through repeated insertion of a needle leads to unnecessary pain and trauma, they require separate connections for joining the primary intravenous line which further complicates intravenous administration, the use of pumps can produce pressures that can vary at the delivery site and the pressure can give rise to thrombosis, the rate of agent delivery to the patient often is unknown as it is not rate-controlled agent delivery but dependent on the flow of fluid, and they often require pre-formulation of the agent medication by the hospital pharmacist or nurse.

In the treatment of patients it is often desirable to provide a means whereby a drug or like agent can be introduced in a controlled manner over an extensive period of time. With the conventional intake of drugs and the like by means of periodic ingestion of a capsule or tablet, or periodic injection, there is usually an initial increase in the amount of the active agent in the body which is then reduced over a period of time, until the next tablet is taken or injection given.

In many cases it is preferable to provide an intake of drug in a controlled manner such that the amount of drug in the body remains substantially constant. This is particularly the case in the treatment of patients suffering constant pain as in the case of terminally ill patients who require a constant amount of an analgesic to be administered in order for them to be able to

withstand the pain.

In the past several devices have been proposed for controlling the introduction of the drug into a body whereby it can be delivered in a controlled manner over a period of time. Such devices have comprised pumps driven by a power source, and which controls the delivery of drug to the body. Some of these pumps may be mounted externally to the body and are connected to a catheter introduced to the body of the patient. Other devices have comprised pump which is mounted subcutaneously to the body of the patient and which delivers a drug to the body at a desired location.

Other delivery devices have comprised a manually operated pump which may be mounted externally to the body or subcutaneously in the body of the patient whereby the pump can be activated by the patient for the delivery of the drug as need arises for that drug. Examples of such devices are disclosed in U.S. Pat. Nos. 4,588,394, 4,681,560 and, 5,085,644, and comprise devices whereby a pumping chamber is connected via catheter directly into the body and derives its source of drug from a holding reservoir.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Example 1

Whole blood was collected in ACD or CPD and kept at 4 C. 50 mL aliquots of the blood were centrifuged in 50 mL conical tubes (1500-1211, USA/Scientific Plastics, Ocala, FL) at 2000 x *g* for 10 minutes, and the plasma and buffy coat were removed together by aspiration. The plasma was then clarified by centrifugation (4000 x *g* for 10 min.) or filtration (160-2045, Nalge Co., Rochester, NY) to remove cells or clots. In cases where the final RBC component was to be stored for more than 24 h, penicillin and streptomycin were added to the plasma to final concentrations of 0.1 percent each.

The RBCs were washed twice, resuspended gently, and then centrifuged again as outlined above. The cells were then similarly washed once in the following IHP solution: 35 mM inositol hexaphosphoric acid, dodecasodium salt (P-8810, Sigma, St. Louis, MO), 33 mM K_2HPO_4, 7 mM NaH_2PO_4, 30.6 mM KCl, 6.4 mM NaCl, 7.3 mM sucrose, and 2 mM ATP (pH 7.2, osmolality 300-325 mmol/kg). With each wash, the residual white blood cells, which formed a buffy coat above the RBCs, were removed with the supernatant by aspiration and

discarded.

A solution consisting essentially of 1 mM IHP, 0.35 mM BGTC, and 3% DMF was sonicated for 15 min. Then, 250 μL of packed human RBCs were incubated with the aforementioned sonicated solution for 60 minutes at 25 min.. After incubation, the RBCs were washed twice by centrifugation at room temperature in 0.9% NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 7 mM sucrose; osmolality 360 mmol/kg. The packed RBCs were then resuspended in bis-Tris buffer (pH 7.3), and the oxy-hemoglobin dissociation curve measured.

A blood-gas monitor (Hemox-Analyzer, TCS Medical Products Co., Huntington Valley, PA) was used to measure IHP internalization in the RBCs on the basis of the P50 shift of the Hb-O_2 dissociation curve. The shift of the P50 values from 22 torr for controls to approximately 35 torr for the incubated RBCs is significant. No changes in the shift were observed when either IHP or the BCTG concentrations were varied. The form of the curve and the Hill coefficients (control: 2.05; RBC-IHP: 1.54-1.58) suggest that the great majority of the incubated RBCs have internalized IHP. The results obtained from this protocol are depicted in Figure 2 [C_a = controls incubated with IHP (1 mM)-DMF (3%); 1a = RBCs + IHP (1 mM)-BGTC (0.35 mM)-DMF (3%); 2a = RBCs + IHP (1 mM)-BGTC (3.5 mmol)-DMF (3%); 3a = IHP (2 mM)-BGTC (0.35 mM)-DMF (3%)].

Incorporation by Reference

All of the patents and publications cited herein are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are encompassed by the following claims.

We claim:

1. A composition consisting essentially of a cationic, lipophilic, water-soluble molecule, and an anionic ligand for a cellular receptor.

2. The composition of claim 1, wherein the anionic ligand is a ligand for the allosteric site of hemoglobin.

3. The composition of claim 2, wherein the anionic ligand is an inositol polyphosphate.

4. The composition of claim 3, wherein the anionic ligand is inositol hexaphosphate.

5. The composition of claim 1, wherein the cationic, lipophilic, water-soluble molecule comprises a guanidinium moiety.

6. The composition of claim 5, wherein the cationic, lipophilic, water-soluble molecule is a sterol comprising at least one guanidinium moiety.

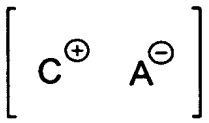
7. The composition of claim 5, wherein the cationic, lipophilic, water-soluble molecule is BGTC or BGSC.

8. The composition of claim 2, wherein the cationic, lipophilic, water-soluble molecule comprises a guanidinium moiety.

9. The composition of claim 3, wherein the cationic, lipophilic, water-soluble molecule is a sterol comprising at least one guanidinium moiety.

10. The composition of claim 4, wherein the cationic, lipophilic, water-soluble molecule is BGTC or BGSC.

11. A compound represented by generalized structure **1**:



1

wherein

C+ represents a lipophilic water-soluble molecule bearing at least one positive charge; and

A- represents a ligand for a mammalian cellular receptor, wherein said ligand bears at least one negative charge.

12. The compound of claim 11, wherein A- is a ligand for the allosteric site of hemoglobin.

13. The compound of claim 11, wherein C+ comprises at least one cationic functional group selected from the group consisting of guanidinium, imidazolium, 1,2-diammoniumethylene, 1,8-diammoniumnaphthyl, and 2,2'-bipyridinium.

14. The compound of claim 11, wherein C+ comprises at least one guanidinium moiety.

15. The compound of claim 11, wherein C+ comprises two guanidinium moieties.

16. The compound of claim 11, wherein C+ comprises at least one cationic functional group selected from the group consisting of guanidinium, imidazolium, 1,2-diammoniumethylene, 1,8-diammoniumnaphthyl, and 2,2'-bipyridinium; and A- is a ligand for the allosteric site of hemoglobin.

17. The compound of claim 11, wherein C+ comprises at least one guanidinium moiety; and A- is a ligand for the allosteric site of hemoglobin.

18. The compound of claim 11, wherein C+ comprises two guanidinium moieties; and A- is a ligand for the allosteric site of hemoglobin.

19. A compound consisting essentially of a lipophilic water-soluble molecule, wherein said lipophilic water-soluble molecule comprises at least one guanidine or guanidinium moiety; and a second molecule comprising at least one carboxylic acid, phosphoric acid, phosphonic acid, sulfuric acid, or sulfonic acid moiety.

20. The compound of claim 19, wherein said second molecule comprises at least one carboxylic acid or phosphoric acid moiety.

21. The compound of claim 19, wherein said second molecule is a phosphorylated inositol.

22. The compound of claim 19, wherein said second molecule is IHP.

23. The compound of claim 19, 20, or 21, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

24. The compound of claim 19 or 20, wherein said lipophilic water-soluble molecule is a sterol.

25. The compound of claim 24, wherein said second molecule is a phosphorylated inositol.

26. The compound of claim 25, wherein said second molecule is IHP.

27. The compound of claim 24, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

28. The compound of claim 25, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

29. The compound of claim 24, wherein said sterol is cholesterol.

30. The compound of claim 25, wherein said sterol is cholesterol.

31. The compound of claim 26, wherein said sterol is cholesterol.

32. The compound of claim 29, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

33. The compound of claim 30, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

34. The compound of claim 24, wherein said lipophilic water-soluble molecule is BGSC or BGTC.

35. The compound of claim 25, wherein said lipophilic water-soluble molecule is BGSC or BGTC.

36. The compound of claim 26, wherein said lipophilic water-soluble molecule is BGSC or BGTC.

37. The compound of claim 34, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

38. The compound of claim 35, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

39. The compound of claim 24, wherein said lipophilic water-soluble molecule is BGTC.

40. The compound of claim 25, wherein said lipophilic water-soluble molecule is BGTC.

41. The compound of claim 26, wherein said lipophilic water-soluble molecule is BGTC.

42. The compound of claim 39, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

43. The compound of claim 40, wherein said second molecule is a ligand for the allosteric site of hemoglobin.

44. A method of enhancing oxygen delivery to a tissue or organ of a mammal, comprising the step of:

administering to said mammal a composition or compound according to claim 1 or 11.

45. A method of enhancing oxygen delivery to a tissue or organ of a mammal, comprising the step of:

administering to said mammal red blood cells previously treated with a composition or compound according to claim 1 or 11.

46. A method of treating a mammal afflicted with anemia, coronary infarction, pulmonary disease, congestive heart failure, myocardial infarction, stroke, peripheral vascular disease, intermittent claudication, circulatory shock, hemorrhagic shock, chronic hypoxia, respiratory alkalemia, metabolic alkalosis, sickle cell anemia, reduced lung capacity, gangrene, anaerobic infections, carbon monoxide poisoning, nitric oxide poisoning, or

cyanide poisoning comprising the step of:

administering to said mammal a composition or compound according to claim 1 or 11.

47. A method of treating a mammal afflicted with anemia, coronary infarction, pulmonary disease, congestive heart failure, myocardial infarction, stroke, peripheral vascular disease, intermittent claudication, circulatory shock, hemorrhagic shock, chronic hypoxia, respiratory alkalemia, metabolic alkalosis, sickle cell anemia, reduced lung capacity, gangrene, anaerobic infections, carbon monoxide poisoning, nitric oxide poisoning, or cyanide poisoning, comprising the step of:

administering to said mammal red blood cells previously treated with a composition or compound according to claim 1 or 11.

48. A method of improving the oxygen delivering capability of mammalian blood, comprising the step of:

adding to said mammalian blood a composition or compound according to claim 1 or 11.

49. A method of incorporating a therapeutically useful substance into mammalian red blood cells, comprising the step of:

treating said mammalian red blood cells with a composition or compound according to claim 1 or 11, wherein said composition or compound comprises said therapeutically useful substance.

Figure 1

Summary of Certain Experiments Forming IHP-BGTC Complexes

1. 0.35 mM BGTC suspension (spin, sonicated, 50 C)
   188 nm particles (measured by light scattering)
   Two populations: i) 56%, 116 nm; ii) 51%, 558 nm

2. 1 mM IHP
   clear solution

3. 1 mM IHP + 0.35 mM BGTC
   (i) precipitation (760 nm-980 nm-1200 nm-1706 nm-2000 nm-2800 nm)
   measurement stopped after five minutes
   (ii) sonicated back to 760 nm, but then particle size increased to 2000 nm
   (iii) 10 μL of serum added, but the size of the particles did not change; subsequent sonication had no discernable effect

4. 2 mL HBSE + 100 μL serum particle size = 930 nm
   (i) addition of BGTC (0.35 mM final concentration): precipitation
   (ii) addition of IHP (1.0 mM final concentration): precipitation, but no greater than without IHP

5. BGTC at 0.35 mM, 3.5 mM, or 35 mM, each with 1 mM IHP
   precipitation (particle size = 800 nm), but 3% DMF limited the particle size to about 480 nm

6. IHP at 1 mM, 2 mM, 5 mM, or 10 mM, with 0.35 mM or 3.5 mM BGTC
   precipitation, but 3% DMF limited the particle size

7. Concentrations described in (6), including DMF; Tris pH 7.1; and washed RBCs, lysed cells, or hemoglobin: precipitation

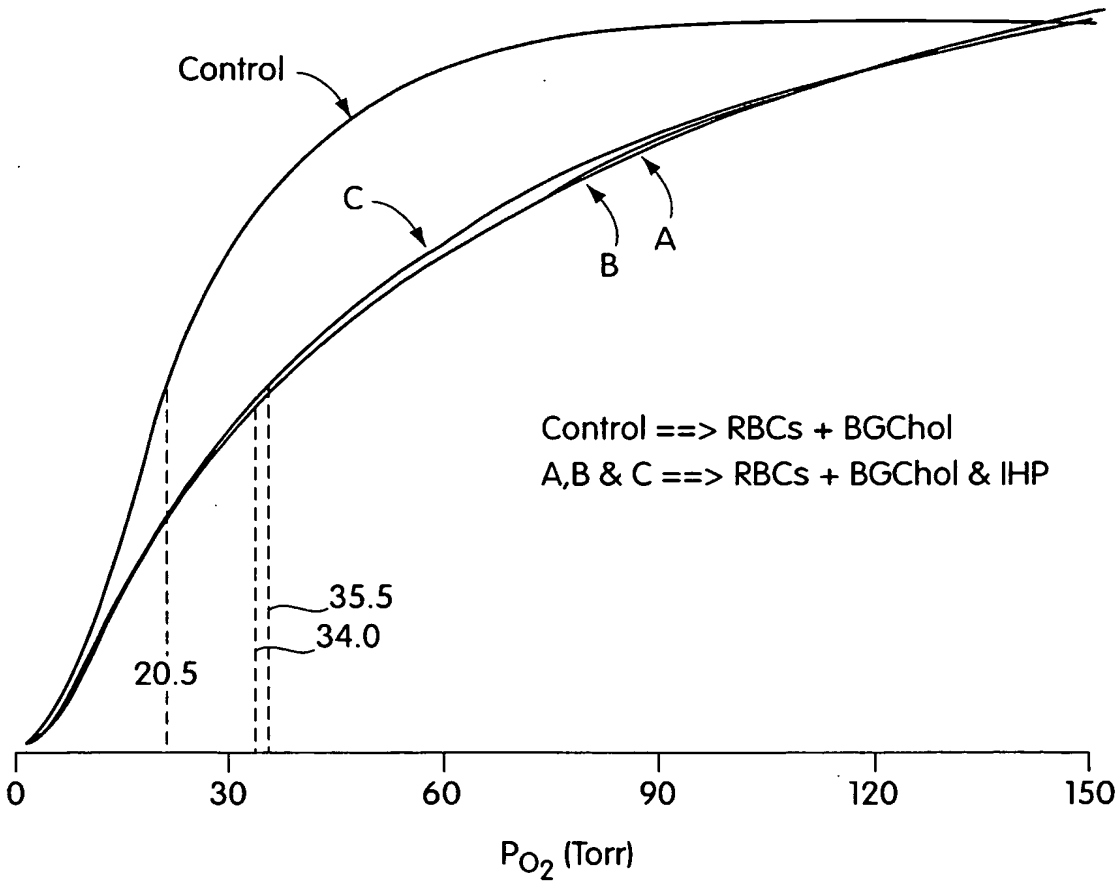

Control
C
B
A
Control ==> RBCs + BGChol
A,B & C ==> RBCs + BGChol & IHP
35.5
34.0
20.5
0
30
60
90
120
150
P_{O_2} (Torr)

Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/22583

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/575 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 97 42819 A (A. HACES) 20 November 1997 (1997-11-20) claims 1-19	1-49
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 January 2001	12.02.2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Herz, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

...ternational application No.
PCT/US 00/22583

B x I Observations where certain claims were found unsearchable (Continuation f item 1 f first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: –
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1 to 49 relate to compounds defined (inter alia) by reference to the following parameters: "a cationic, lipophilic, water-soluble molecule" and "an anionic ligand for a cellular receptor". The use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible. Consequently, the search has been restricted to the compounds BGTC, BGSC and IHP.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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